FIGURE 1

Immunoaffinity Isolation of Modified Peptides
From Complex Mixtures
Rush, et al
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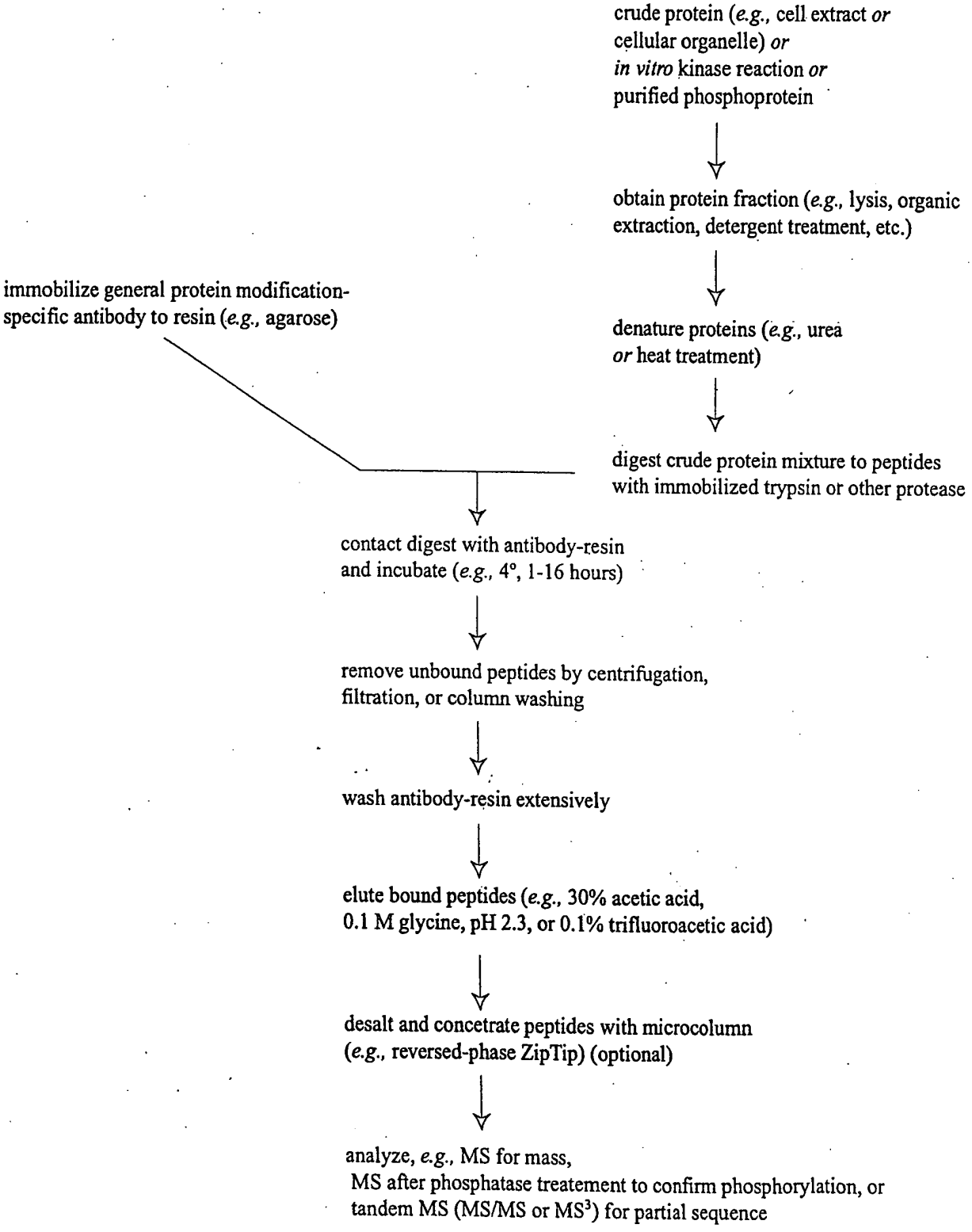

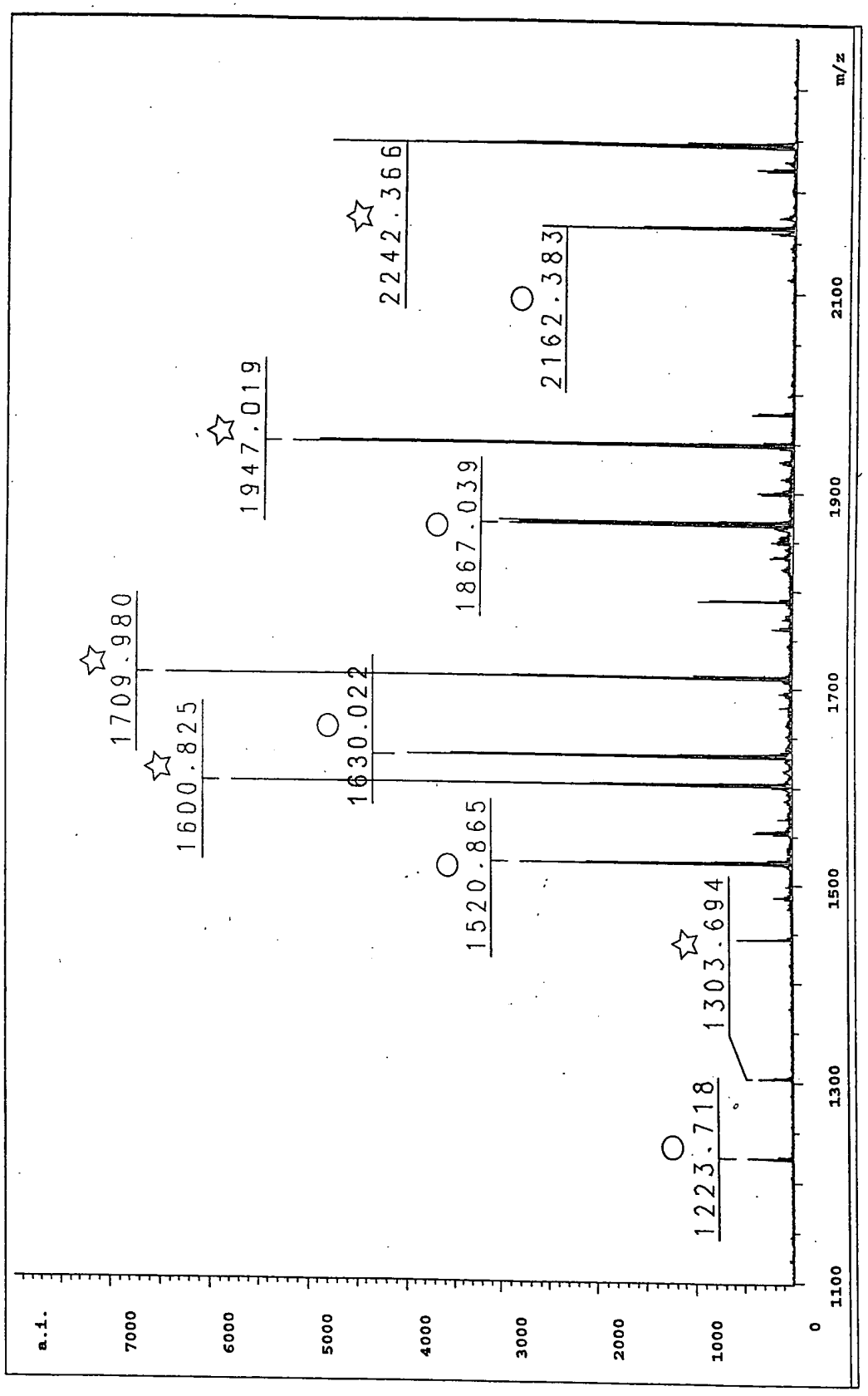

FIGURE 2: MALDI-TOF mass spectrum of an unpurified phosphotyrosine peptide mix.

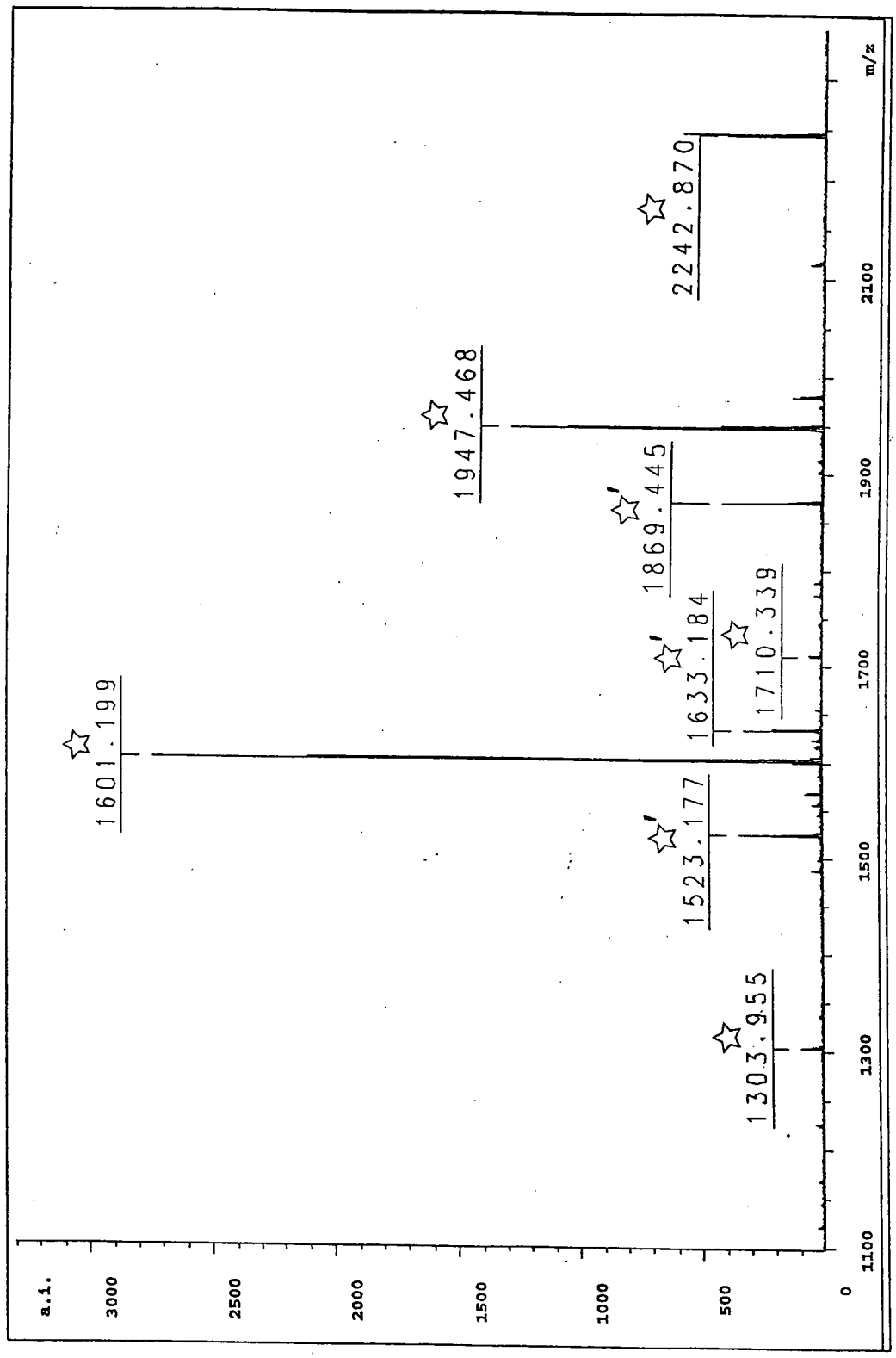

FIGURE 3: MALDI-TOF mass spectrum of a phosphotyrosine peptide mix after purification with P-Tyr-100 monoclonal antibody.

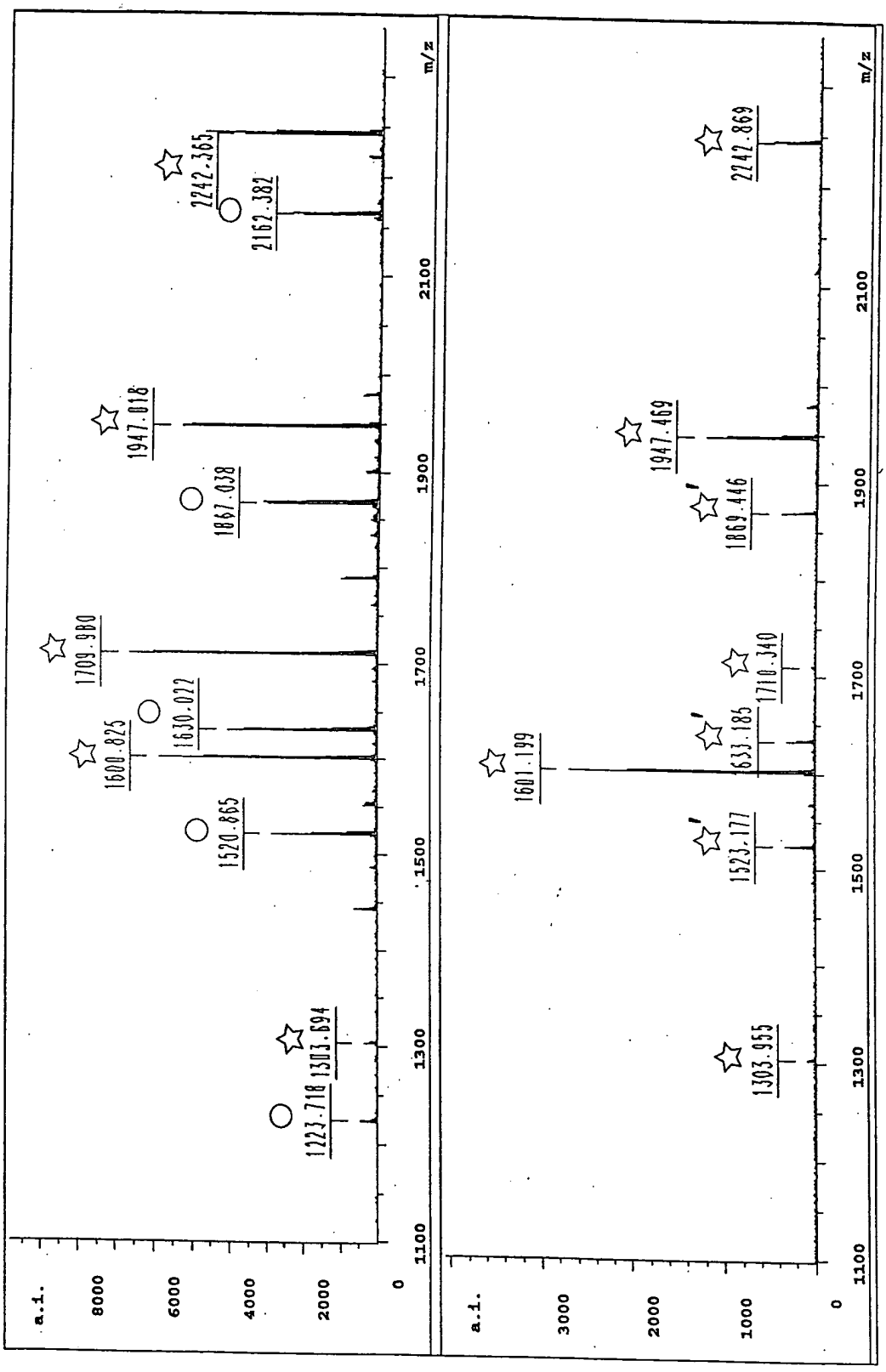

FIGURE 4: MALDI-TOF mass spectra of a phosphotyrosine peptide mix before and after purification with P-Tyr-100 monoclonal antibody.

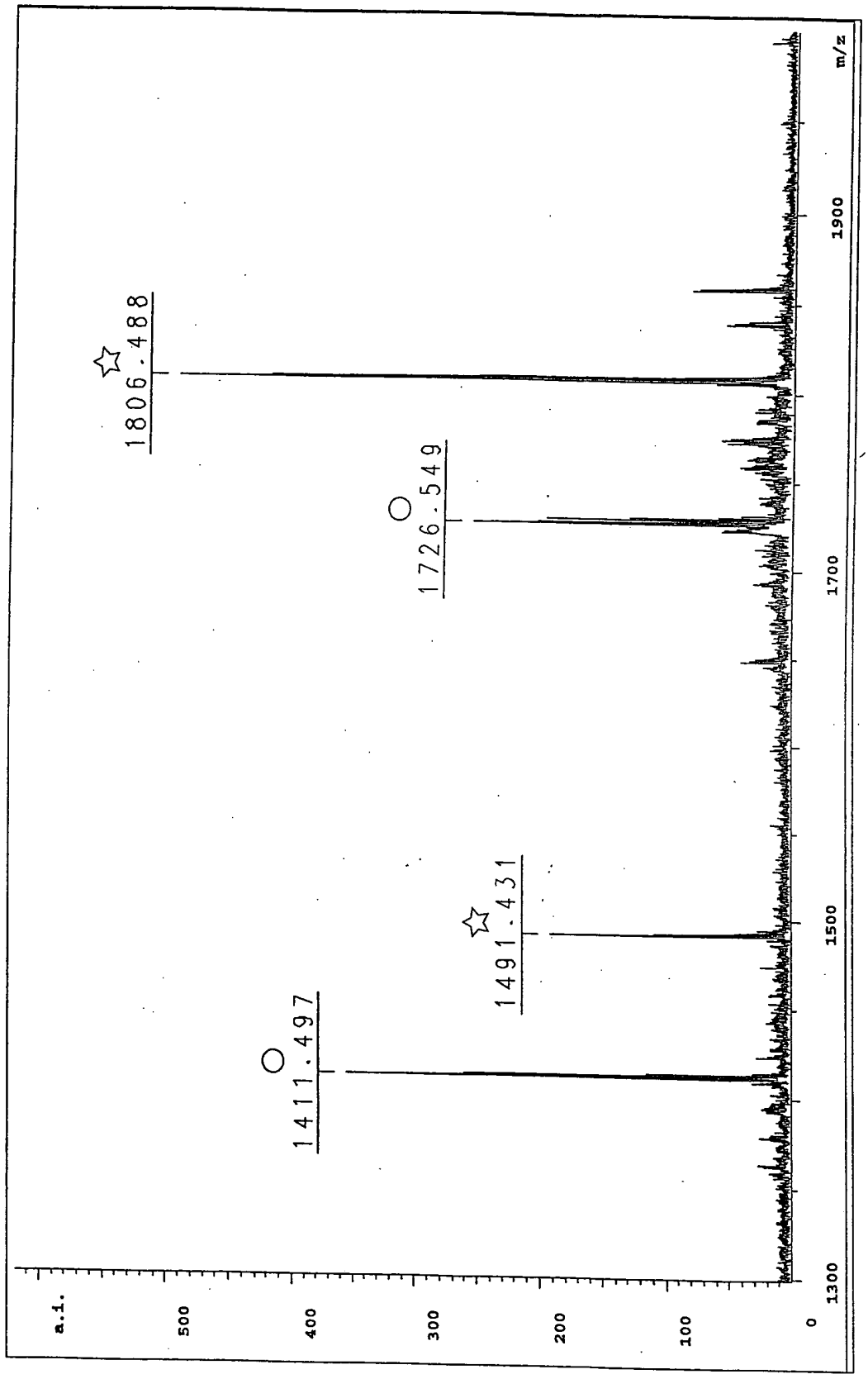

FIGURE 5: MALDI-TOF mass spectrum of an unpurified phosphothreonine peptide mix.

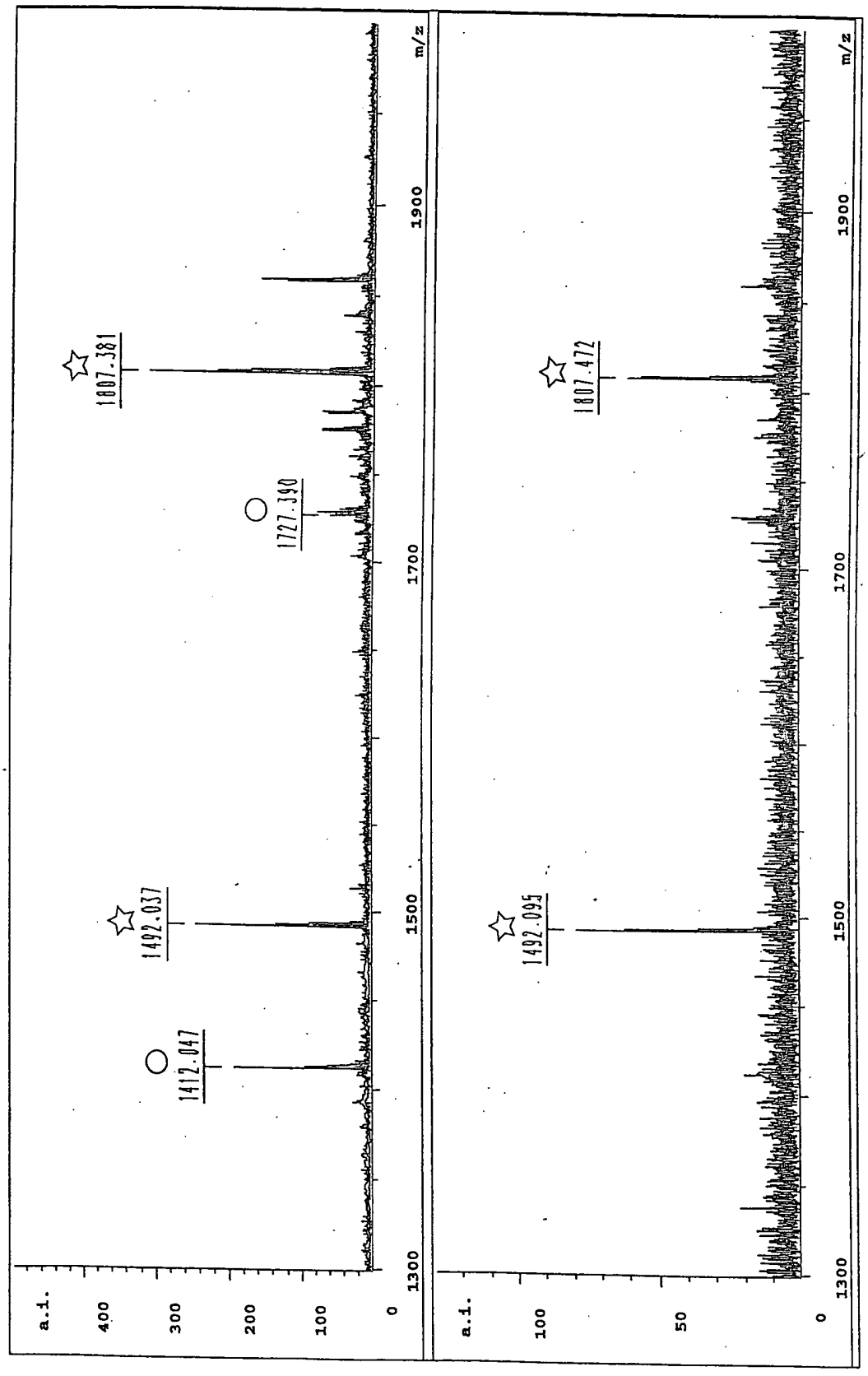

FIGURE 6: MALDI-TOF mass spectra of a phosphothreonine peptide mix after purification with P-Thr polyclonal antibody, showing the unbound peptide fraction and the bound and eluted peptide fraction.

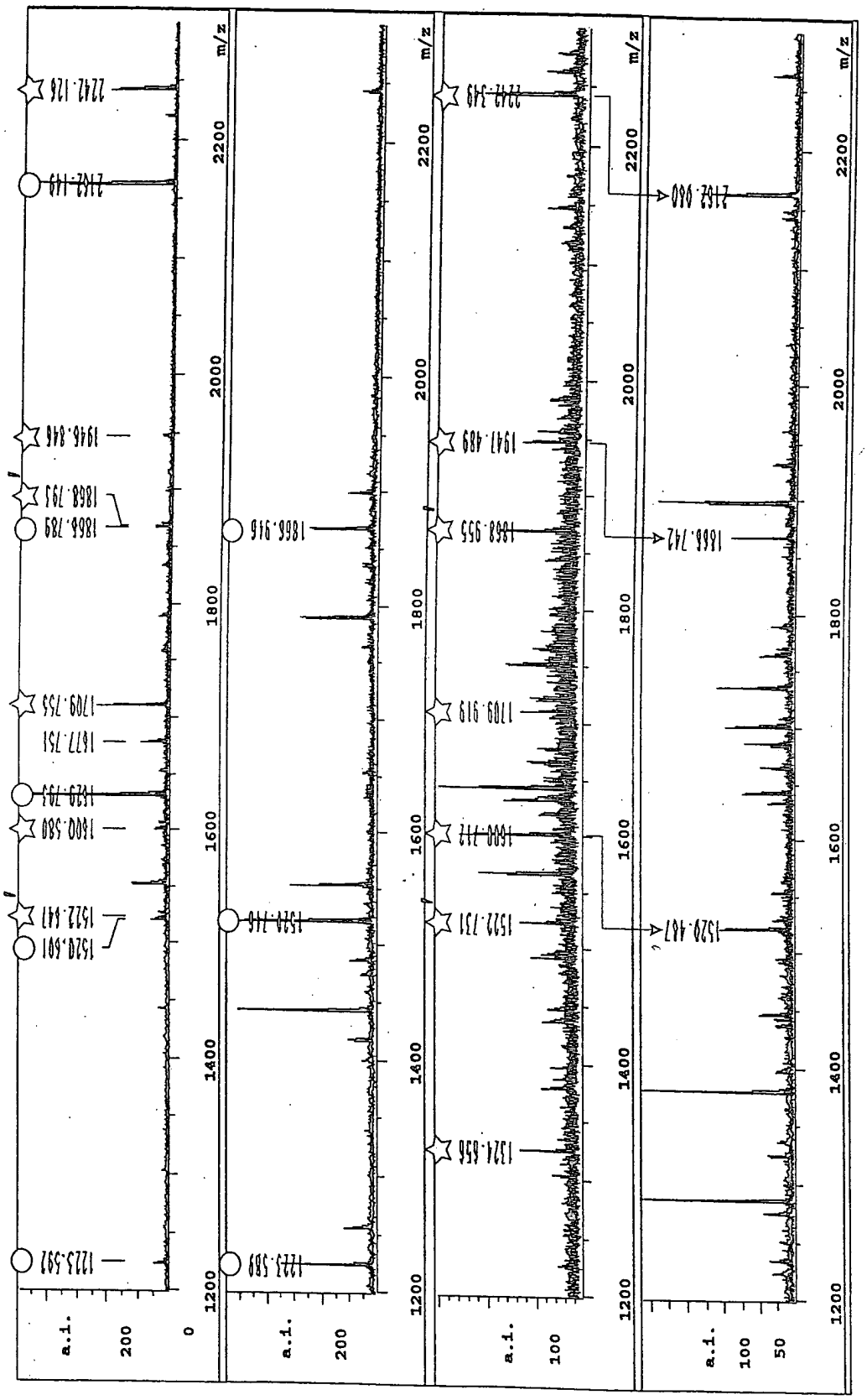

FIGURE 7: MALDI-TOF mass spectra of a phosphotyrosine peptide mix before (panel 1) and after (panels 2-4) purification at low levels (<1 pmol) with P-Tyr-100 monoclonal antibody.

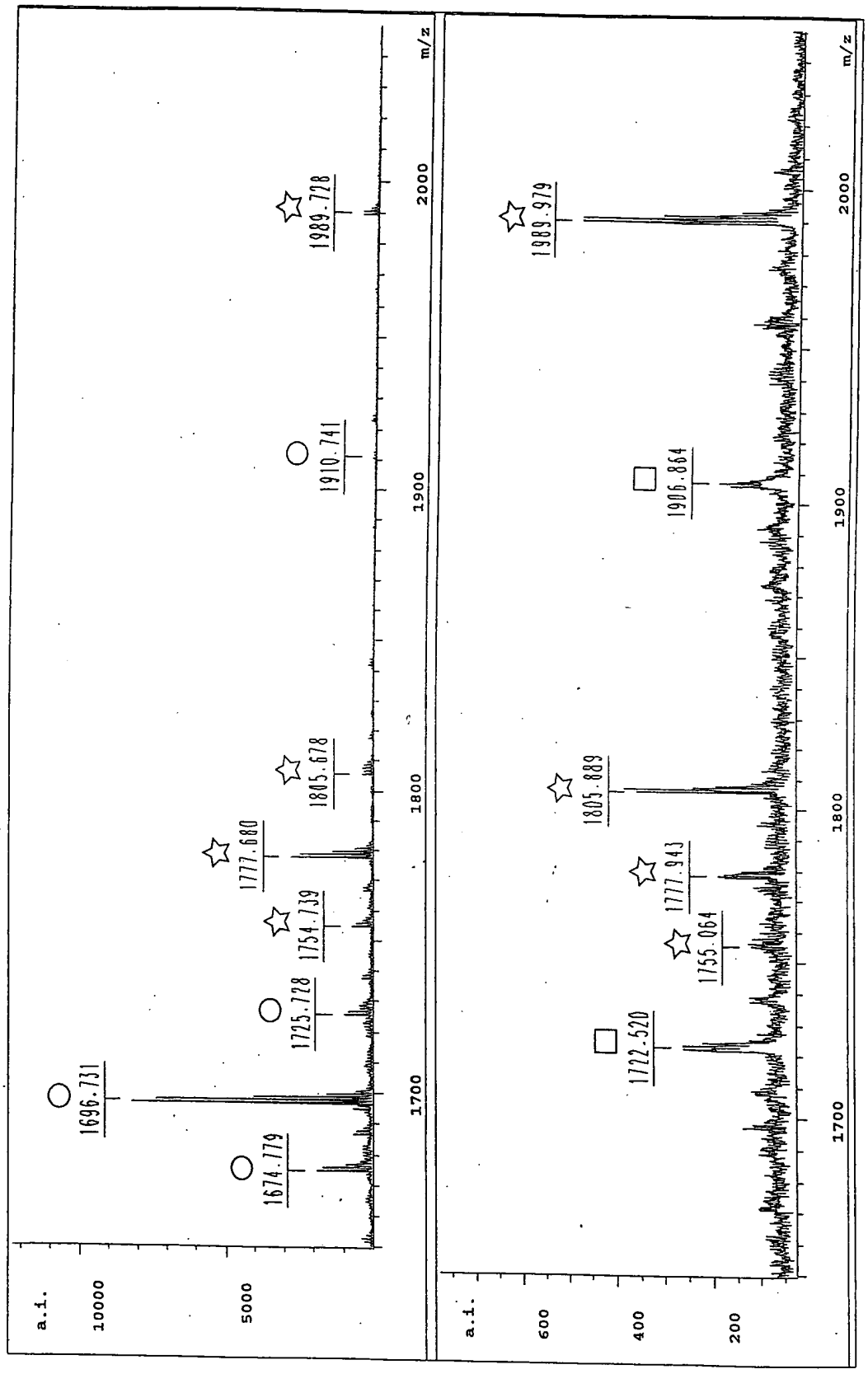

FIGURE 8: MALDI-TOF mass spectra of a phospho-Akt substrate peptide mix before and after purification with phospho-(Ser/Thr) Akt substrate polyclonal antibody.

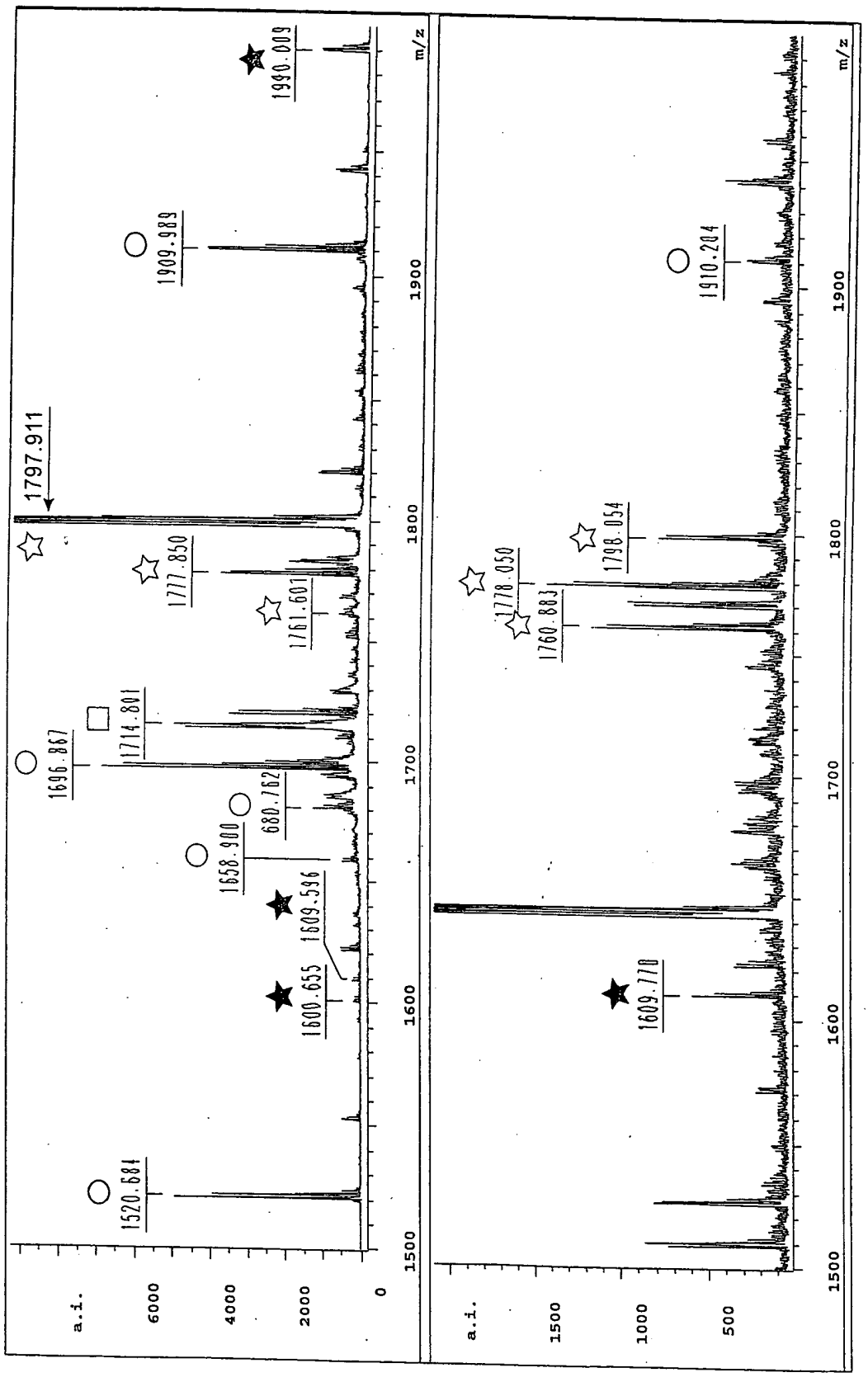

FIGURE 9: MALDI-TOF mass spectra of a 14-3-3 binding motif peptide mix before and after purification with phospho-(Ser) 14-3-3 binding motif monoclonal antibody.

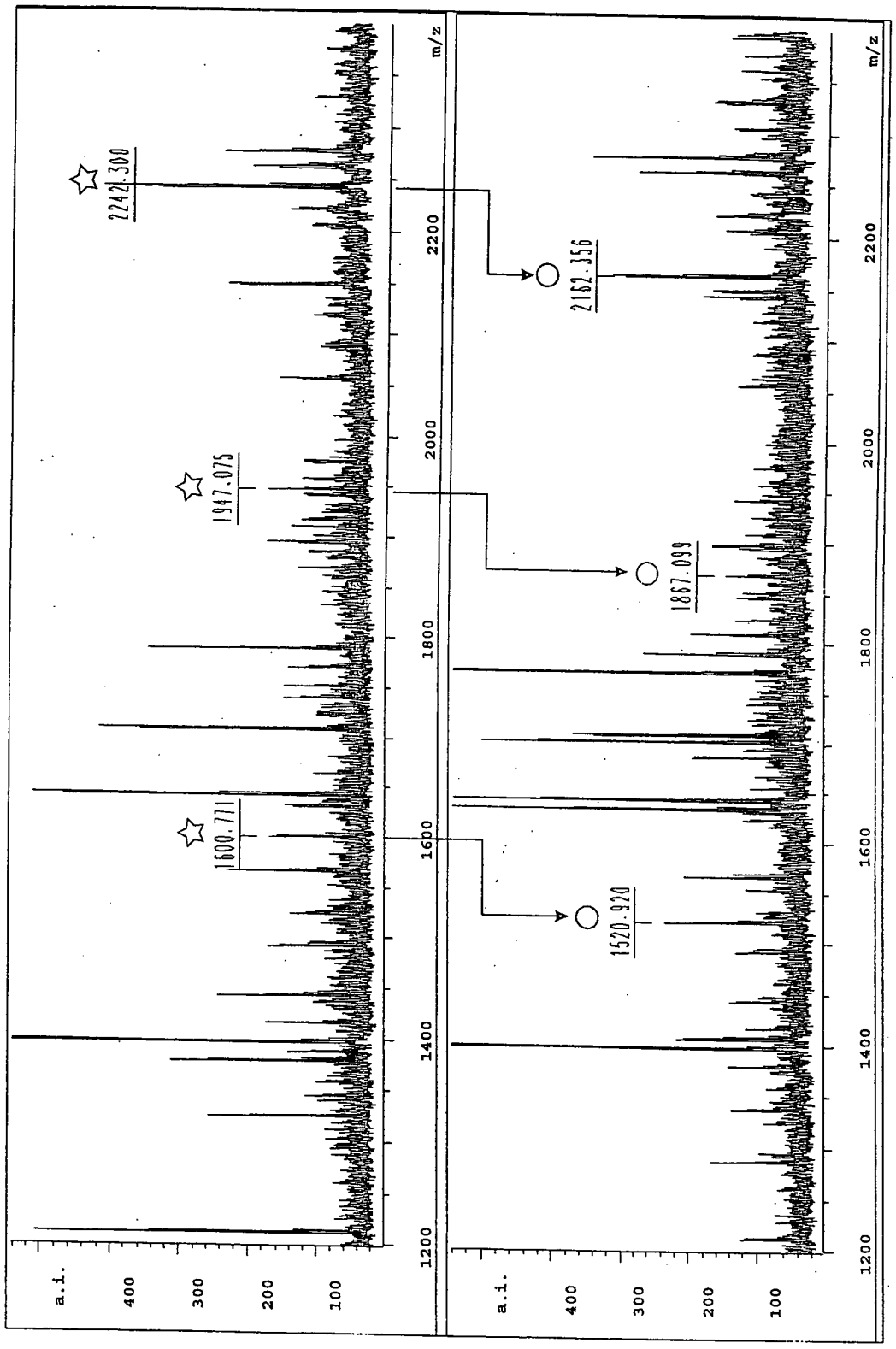

FIGURE 10: MALDI-TOF mass spectra of the bound and eluted peptide fraction purified from a mixture of digested cell extract, phosphotyrosine peptide mix, and phospho-Akt substrate peptide mix with P-Tyr-100 monoclonal antibody, before and after phosphatase treatment.

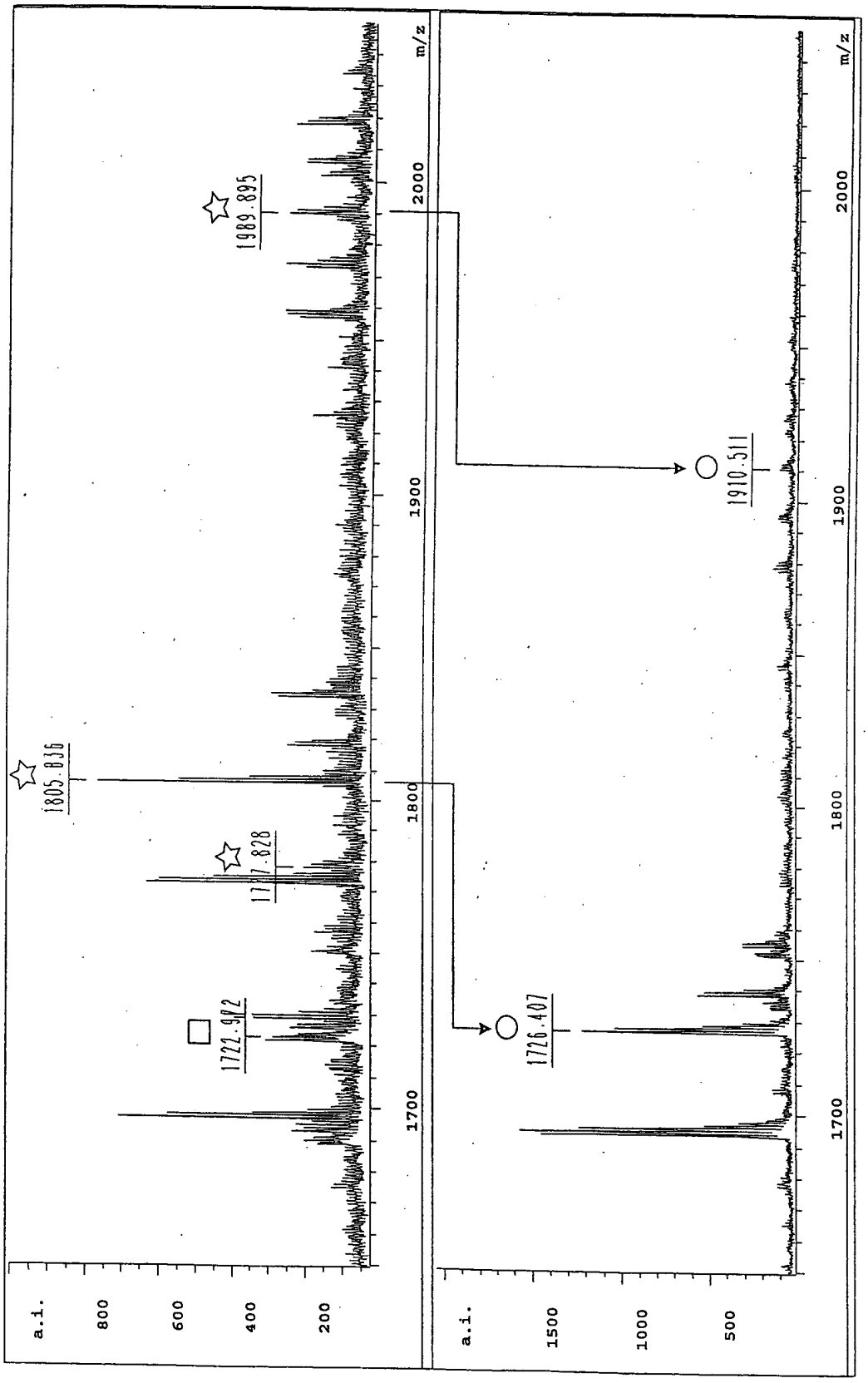

FIGURE 11: MALDI-TOF mass spectra of the bound and eluted peptide fraction purified from a mixture of digested cell extract, phosphotyrosine peptide mix, and phospho-Akt substrate peptide mix with phospho-(Ser/Thr) Akt substrate polyclonal antibody, before and after phosphatase treatment.

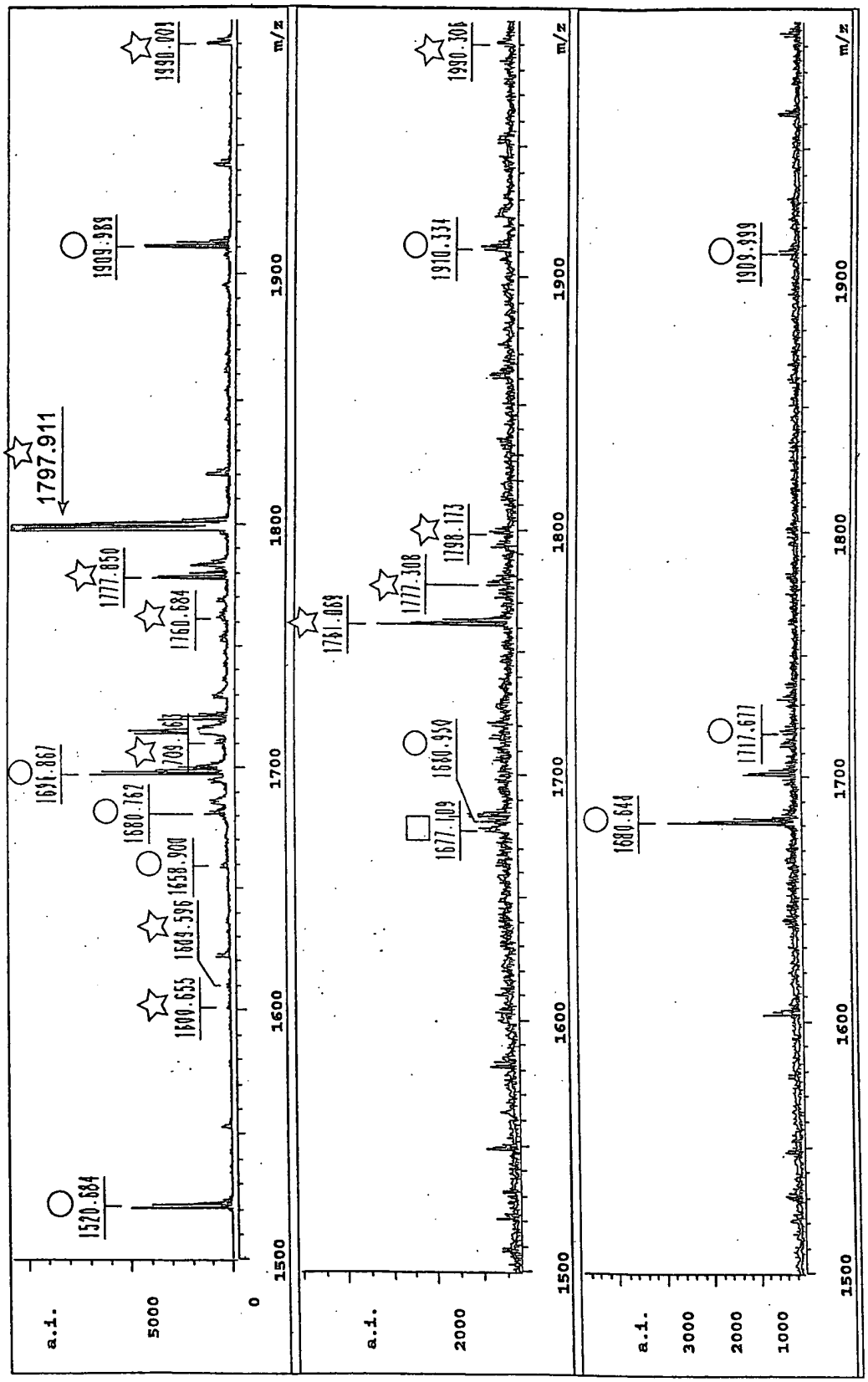

FIGURE 12: MALDI-TOF mass spectrum of the bound and eluted peptide fraction purified from a mixture of digested cell extract and 14-3-3 binding motif peptide mix with phospho-(Ser) 14-3-3 binding motif monoclonal antibody, before and after phosphatase treatment.

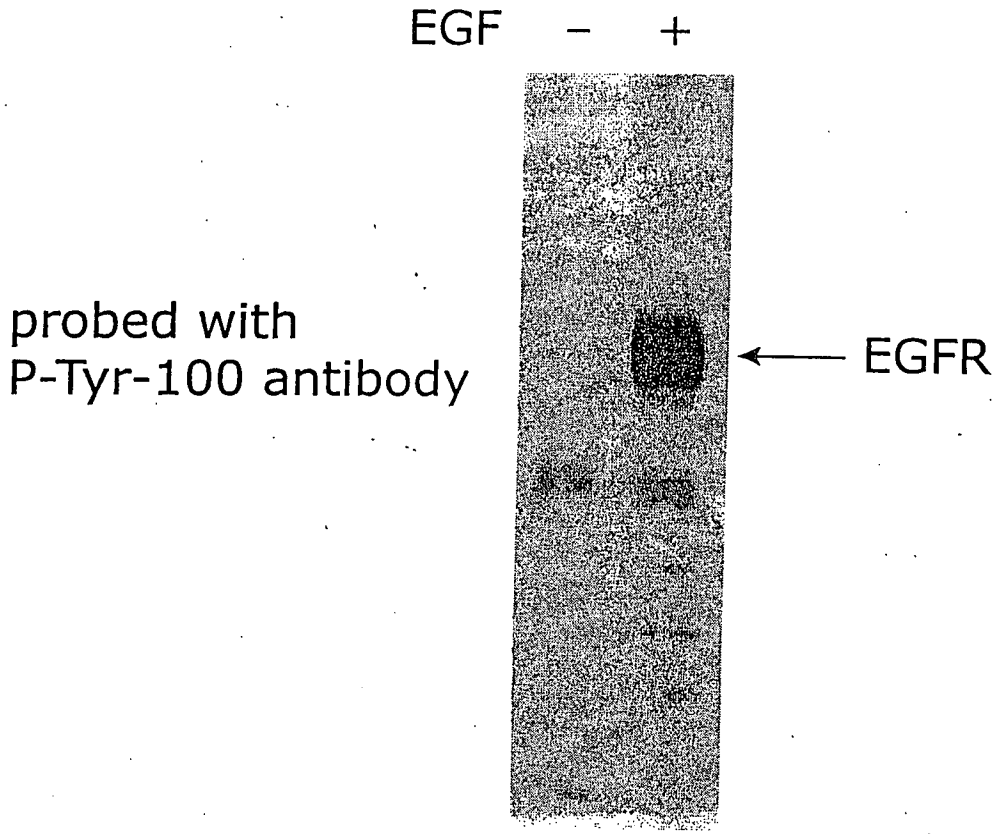

FIGURE 13: Western blot of A431 cells overexpressing the epidermal growth factor receptor (EGFR) and probed with P-Tyr-100 monoclonal antibody.

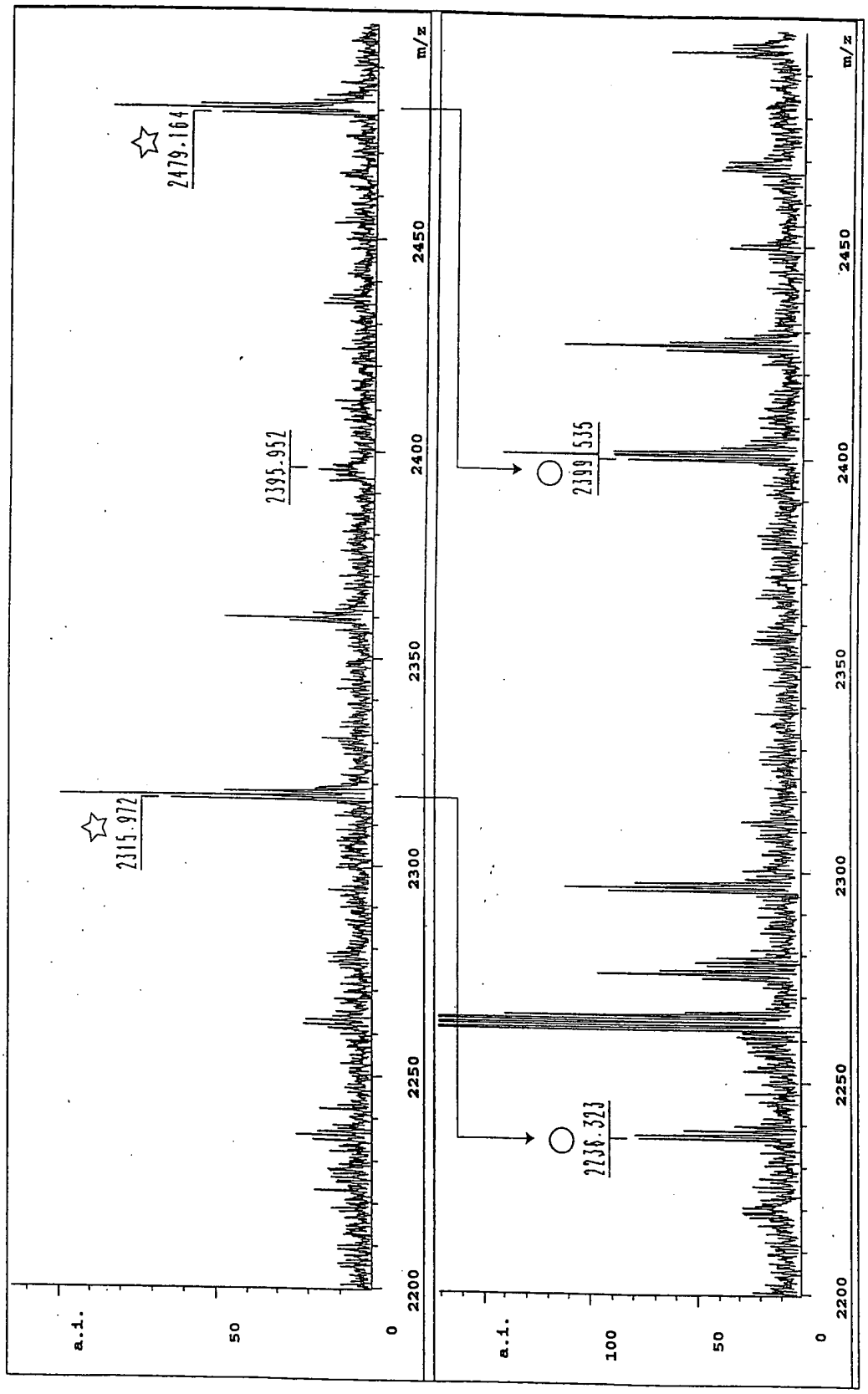

FIGURE 14: MALDI-TOF mass spectra of the bound and eluted peptide fraction purified from a digested extract of A431 cells overexpressing EGFR with P-Tyr-100 monoclonal antibody, before and after phosphatase treatment.

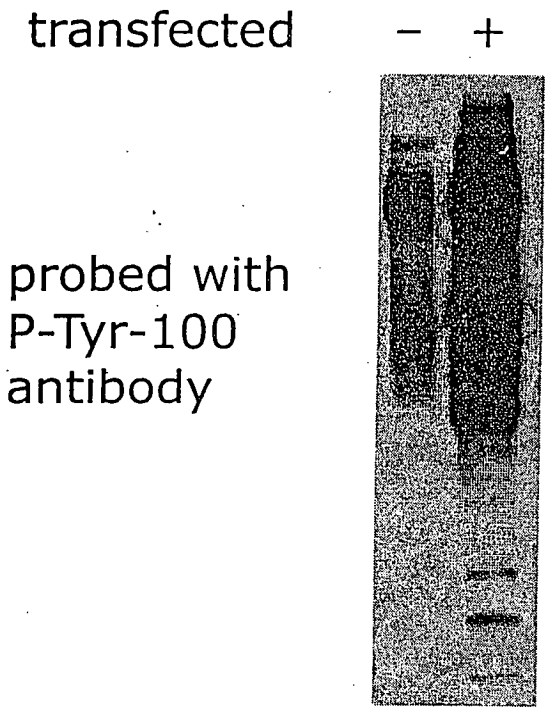

FIGURE 15: Western blot of 3T3 cells transfected to express active Src protein kinase constituitively and probed with P-Tyr-100 monoclonal antibody.

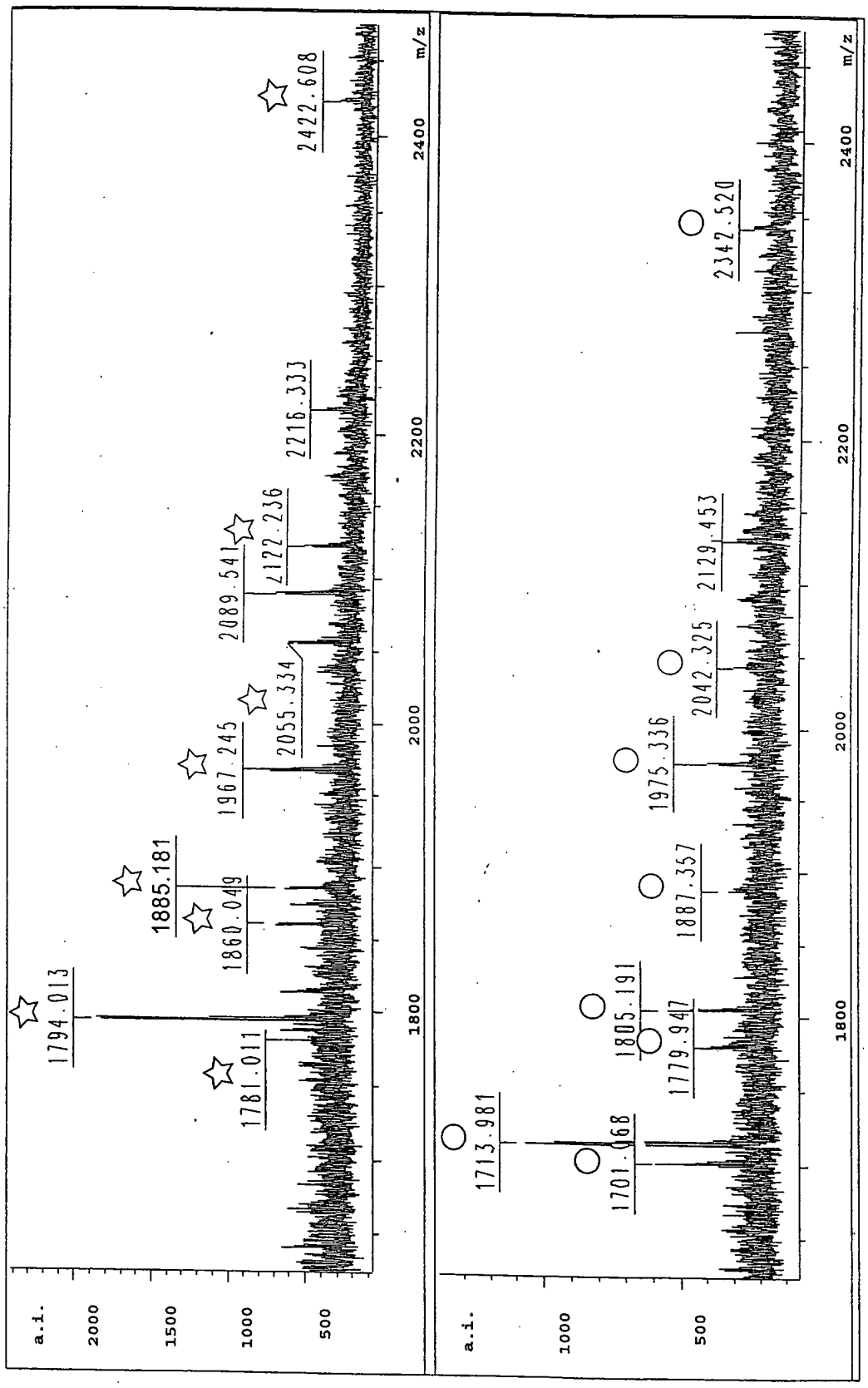

FIGURE 16: MALDI-TOF mass spectra of the bound and eluted peptide fraction purified from a digested extract of 3T3 cells transfected to express active Src protein kinase constituitively with P-Tyr-100 monoclonal antibody, before and after phosphatase treatment.

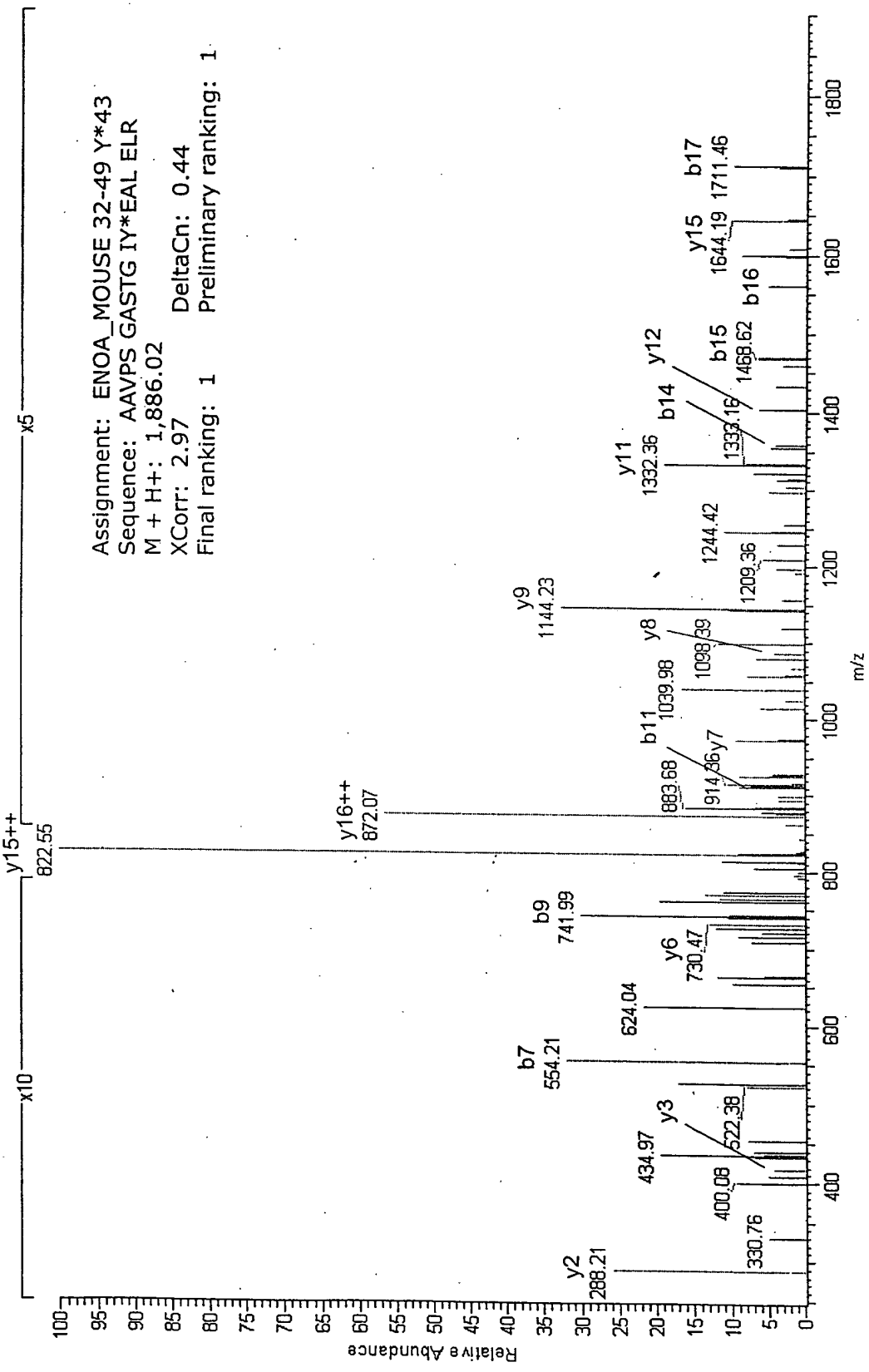

FIGURE 17: LC-MS/MS spectrum of one of the modified peptides purified from an extract of 3T3 cells transfected with Src protein kinase with immobilized P-Tyr-100 antibody.

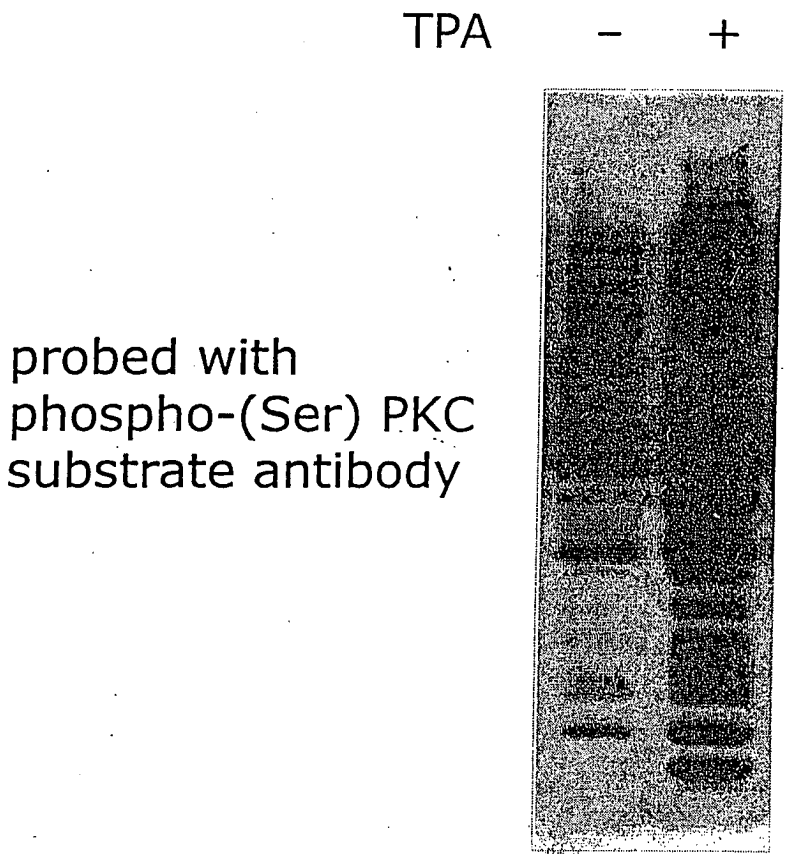

FIGURE 18: Western blot of Jurkat cells treated with TPA and probed with phospho-(Ser) PKC substrate antibody.

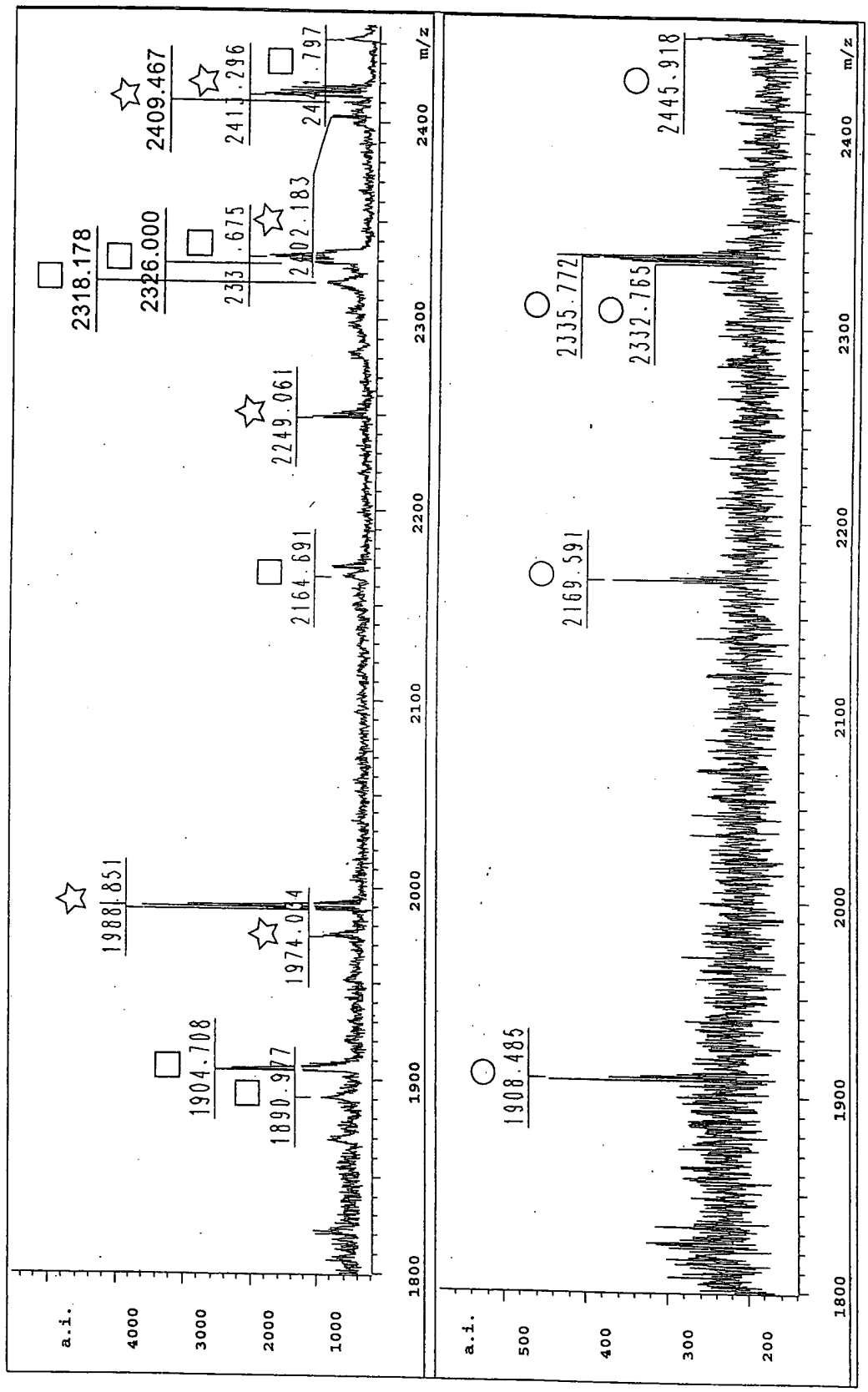

FIGURE 19, PANEL A: MALDI-TOF mass spectrum of modified peptides isolated from a TPA-treated Jurkat cell extract with immobilized phospho-PKC substrate antibody, before and after phosphatase treatment.

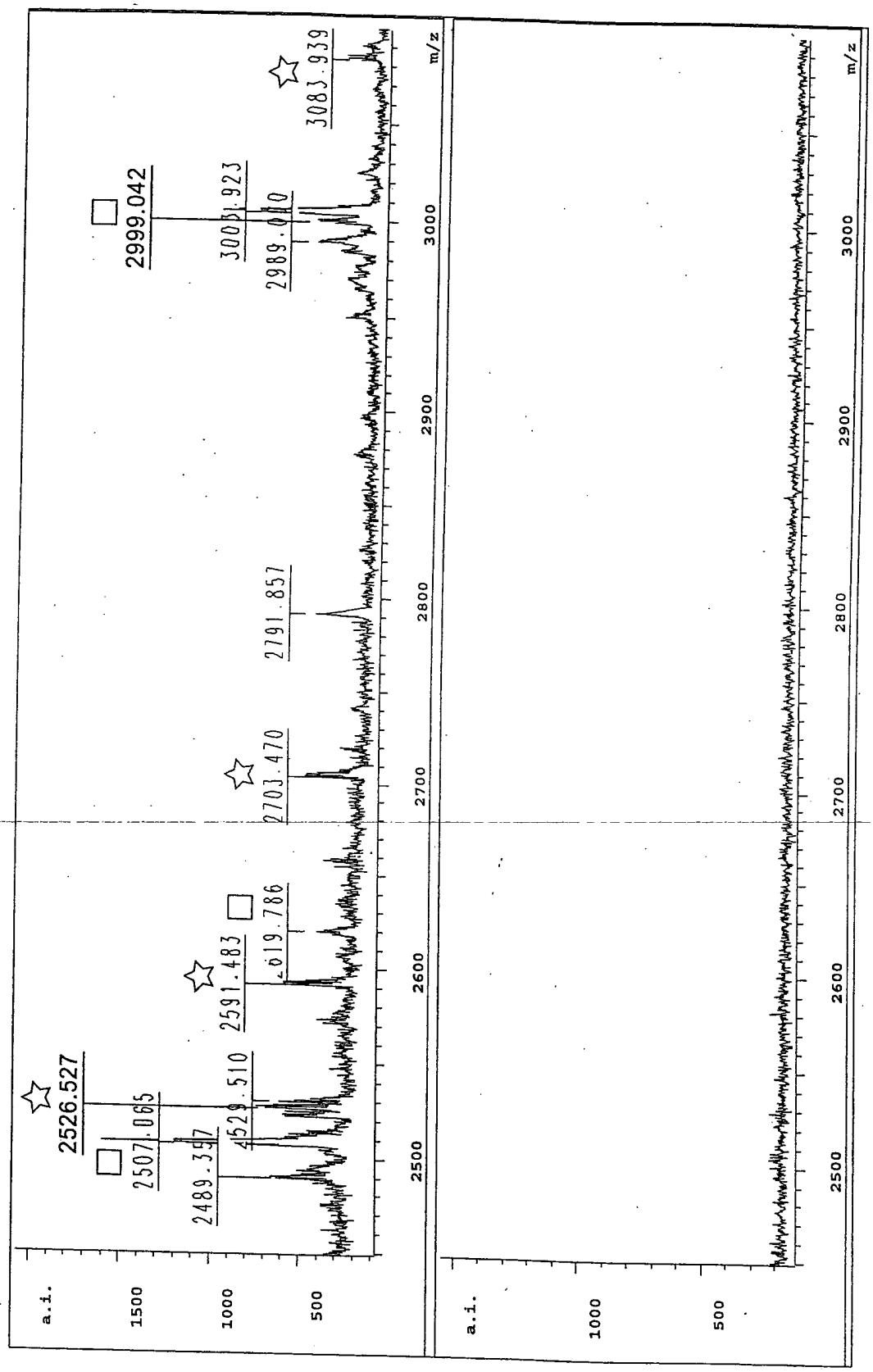

FIGURE 19, PANEL B: MALDI-TOF mass spectrum of modified peptides isolated from a TPA-treated Jurkat cell extract with immobilized phospho-PKC substrate antibody, before and after phosphatase treatment.

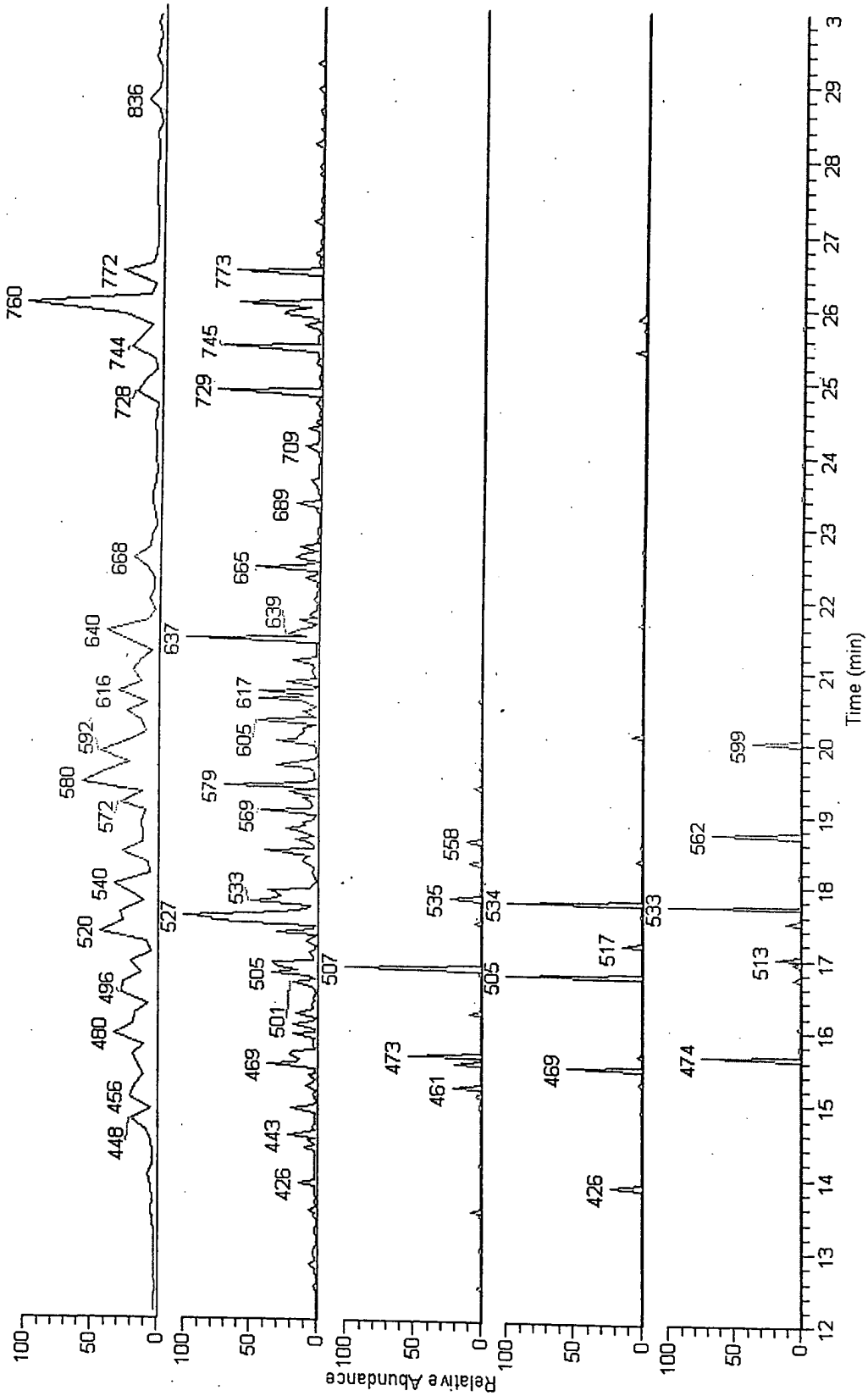

FIGURE 20: Various chromatograms obtained by LC-MS/MS analysis of the modified peptides isolated from a TPA-treated Jurkat cell extract using immobilized phospho-PKC substrate antibody.

LC-MS/MS Spectrum No.	m/z	z	m	Number of Phosphates	Seen by MALDI	Label in MALDI	Identified by MS^3 as protein residues site
579	583.770	2	1,165.526	1	~		
417	668.300	2	1,334.586	1	~		UFD1 333-343 S*335
426	446.030	3	1,335.069	1	~		as above
461	953.620	2	1,905.226	1	~		
470	995.100	2	1,988.186	1	+	1,988.851	
469	664.000	3	1,988.979	1	+	as above	
473	1,205.480	2	2,408.946	1	+	2,409.467	PTN6 576-595 S*588
474	603.640	4	2,410.532	1	+	as above	as above
535	1,207.650	2	2,413.286	1	+	2,413.296	UFD1 322-343 S*335
534	805.310	3	2,412.909	1	+	as above	as above
533	604.440	4	2,413.732	1	+	as above	as above
558	1,209.750	2	2,417.486	1	~		
507	1,297.140	2	2,592.266	1	+	2,591.483	
505	865.040	3	2,592.099	1	+	as above	
513	807.900	4	3,227.572	1	-		
599	824.250	4	3,292.972	1	~		
601	1,099.170	3	3,294.489	1	~		
562	1,018.680	4	4,070.692	1	-		

For "Seen in MALDI", + indicates yes, ~ possibly (obscurred or sometimes seen), - no.
Note masses above 3,600 were not measured during MALDI-TOF mass spectrometry.

FIGURE 21: Properties of the peptides that were observed to undergo neutral-loss during the LC-MS/MS analysis shown in Figure 20.

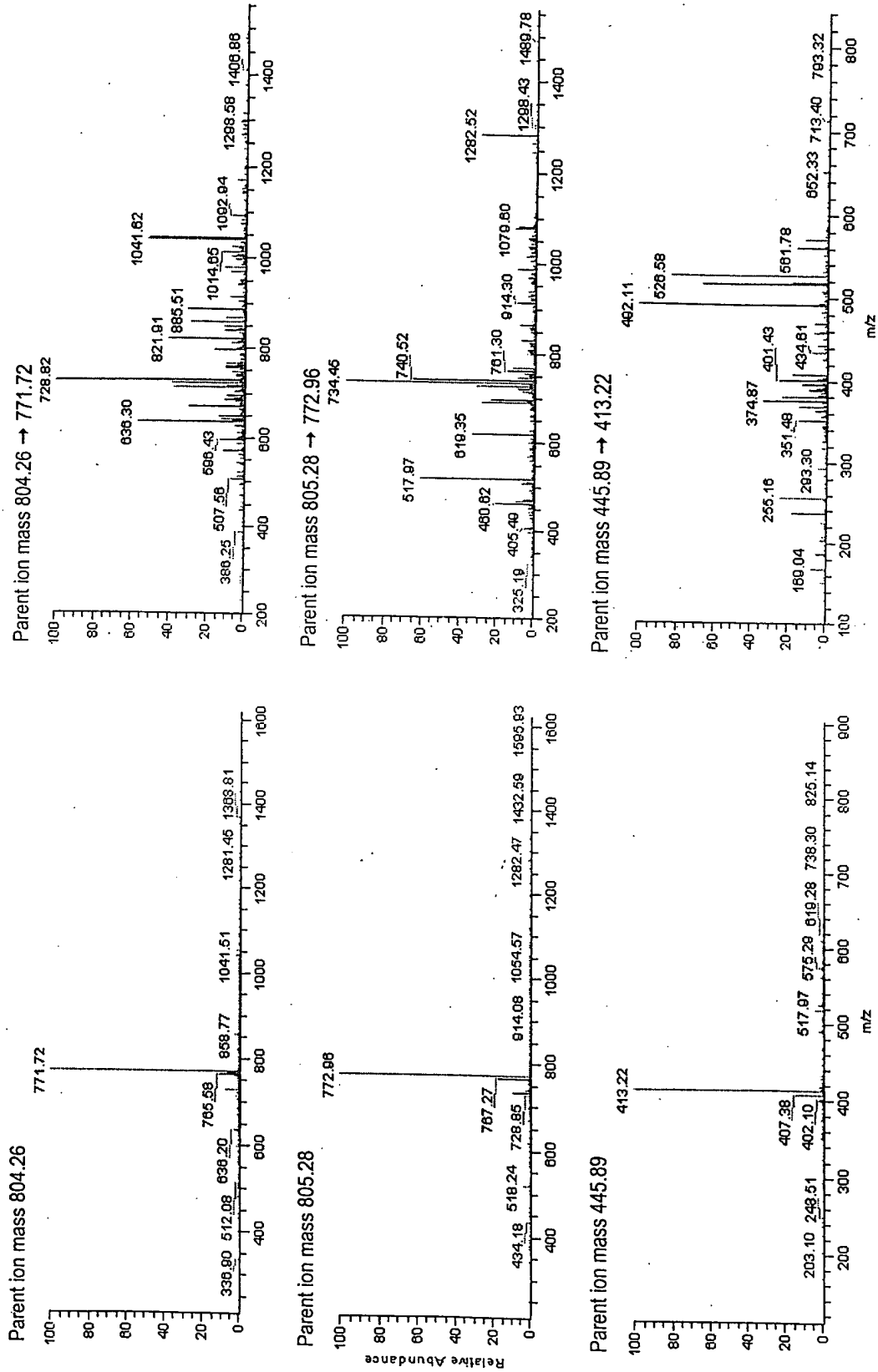

FIGURE 22: Some of the MS/MS spectra (left panels) and MS3 spectra (right panels) acquired during LC-MS3 analysis of the modified peptides purified from a TPA-treated Jurkat cell extract with immobilized phospho-PKC substrate antibody.

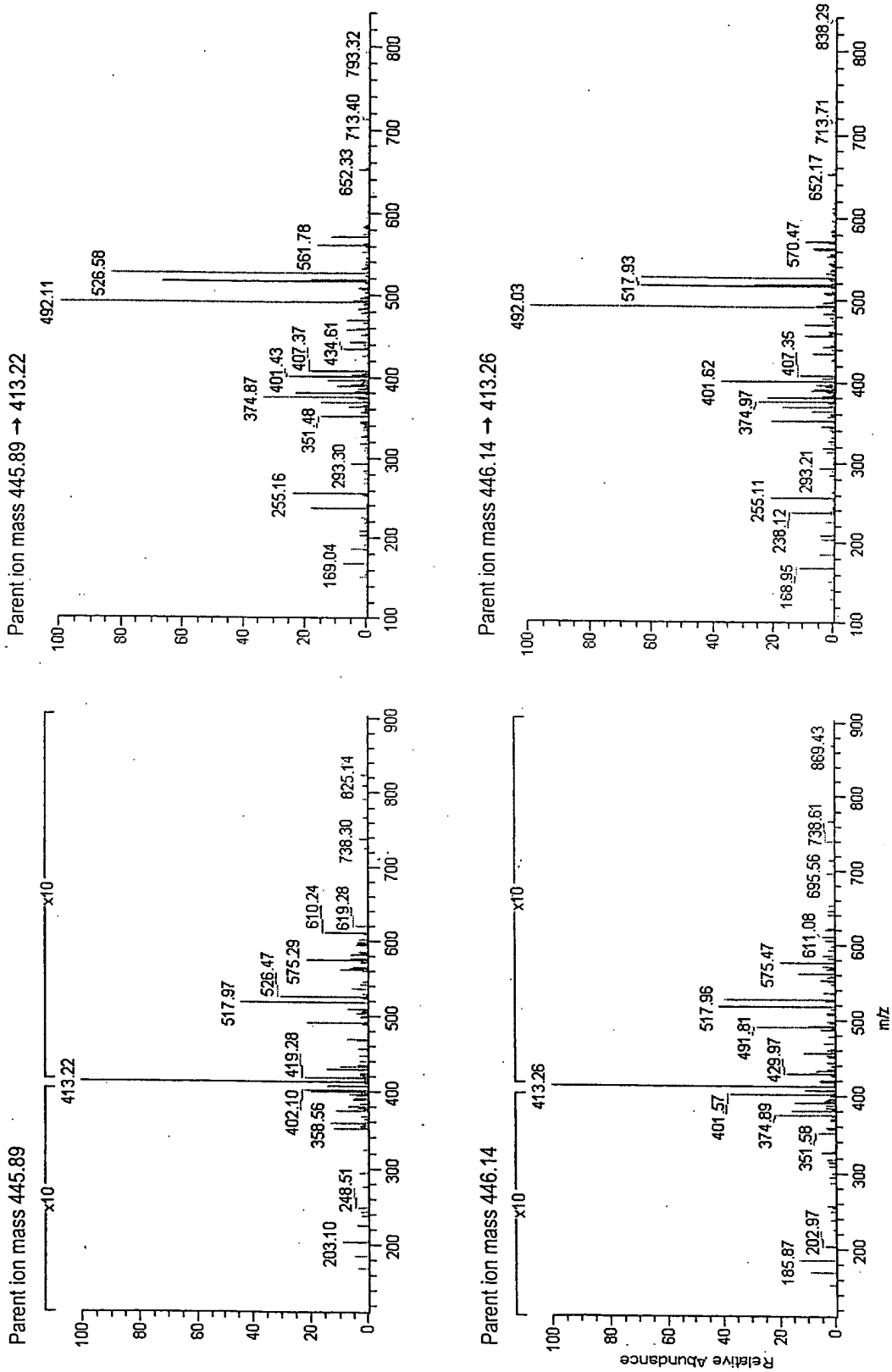

FIGURE 23: MS/MS spectra (left panels) and MS3 spectra (right panels) that confirm an assignment made by Sequest.

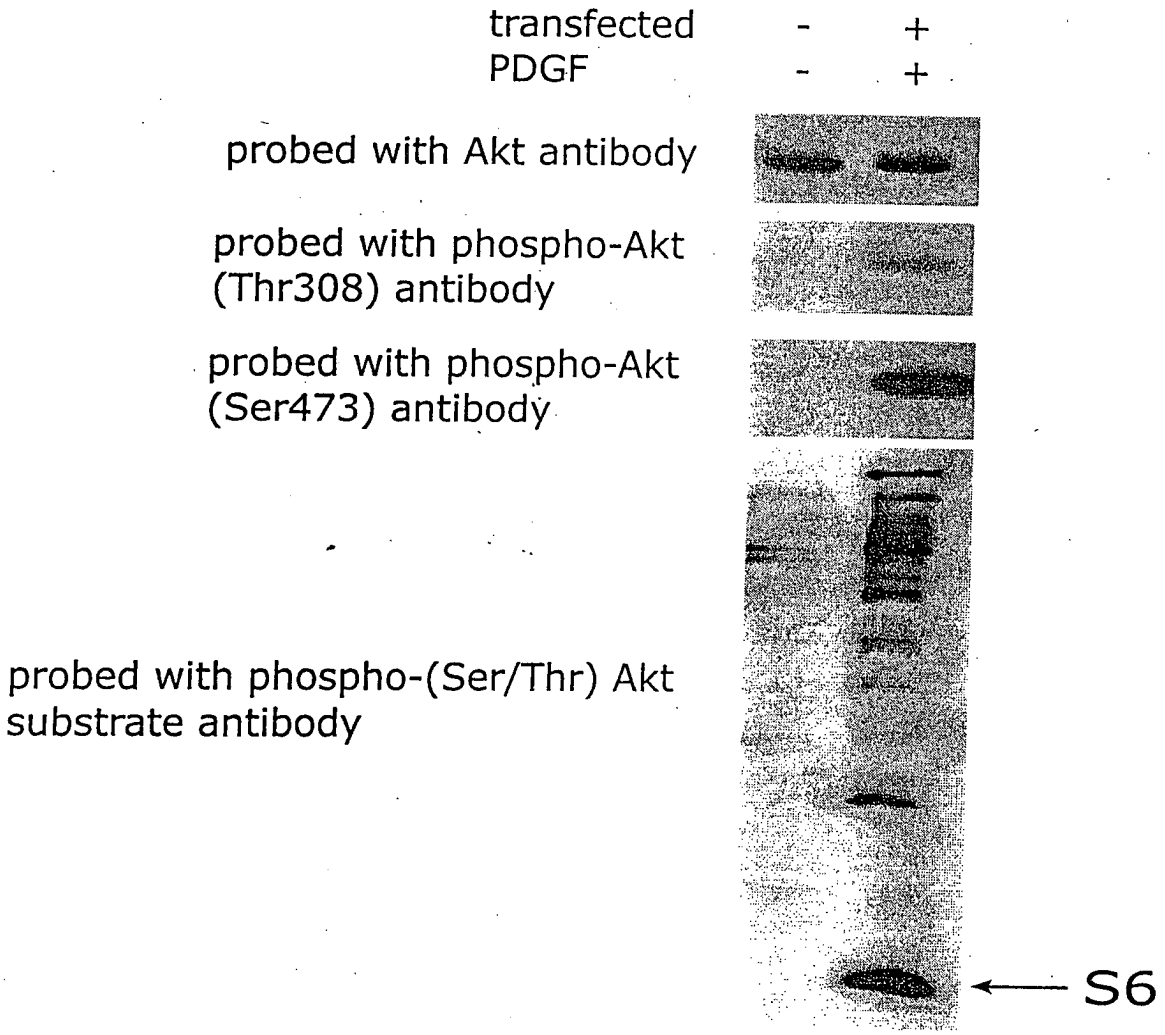

FIGURE 24: Western blot of 3T3 cells transfected to express active Akt protein kinase constituitively and probed with various Akt antibodies or with phospho-(Ser/Thr) Akt substrate polyclonal antibody.

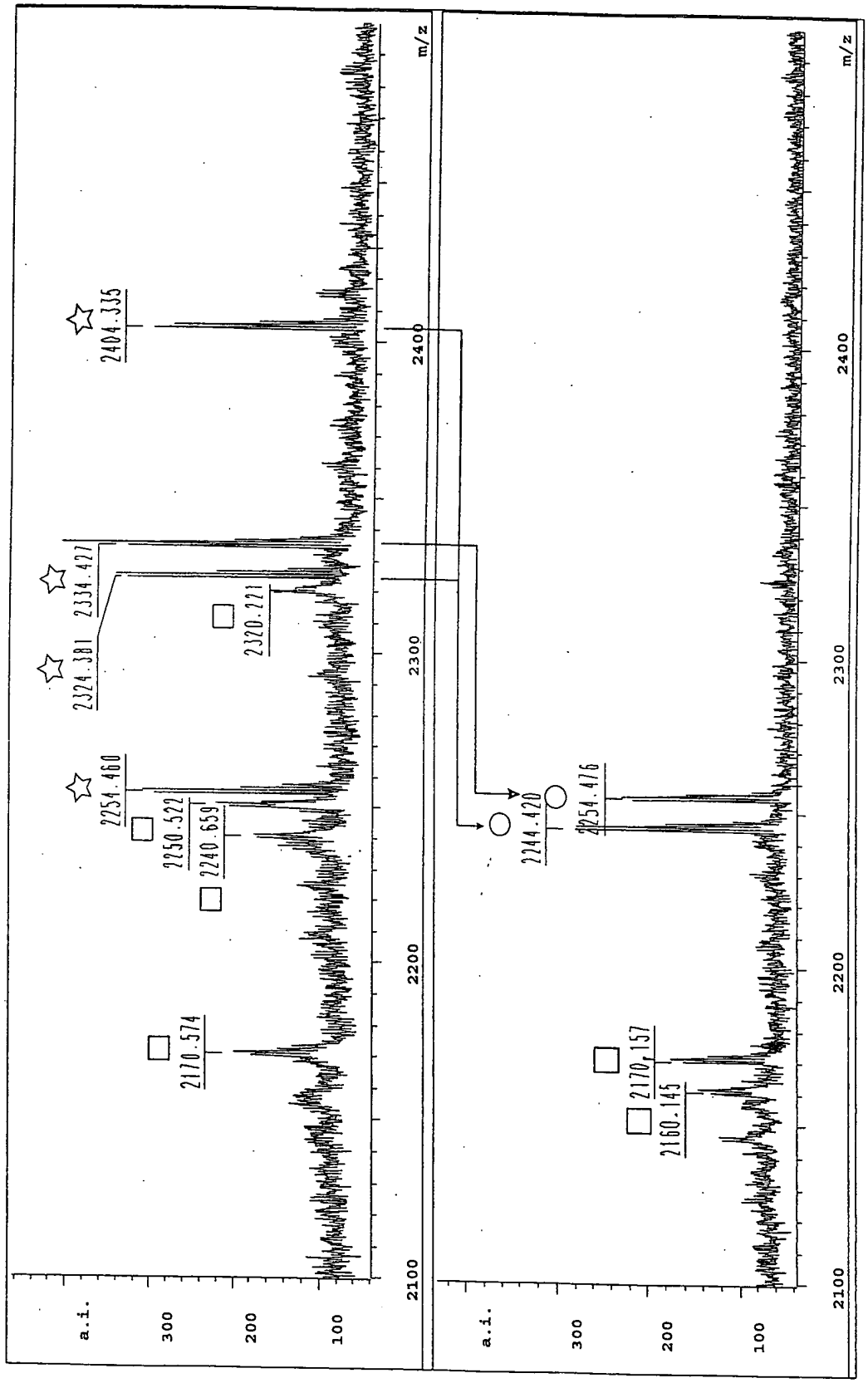

FIGURE 25: MALDI-TOF mass spectra of the bound and eluted peptide fraction purified from a digested extract of 3T3 cells transfected to express active Akt protein kinase constitutively with phospho-(Ser/Thr) Akt substrate polyclonal antibody, before and after phosphatase treatment.

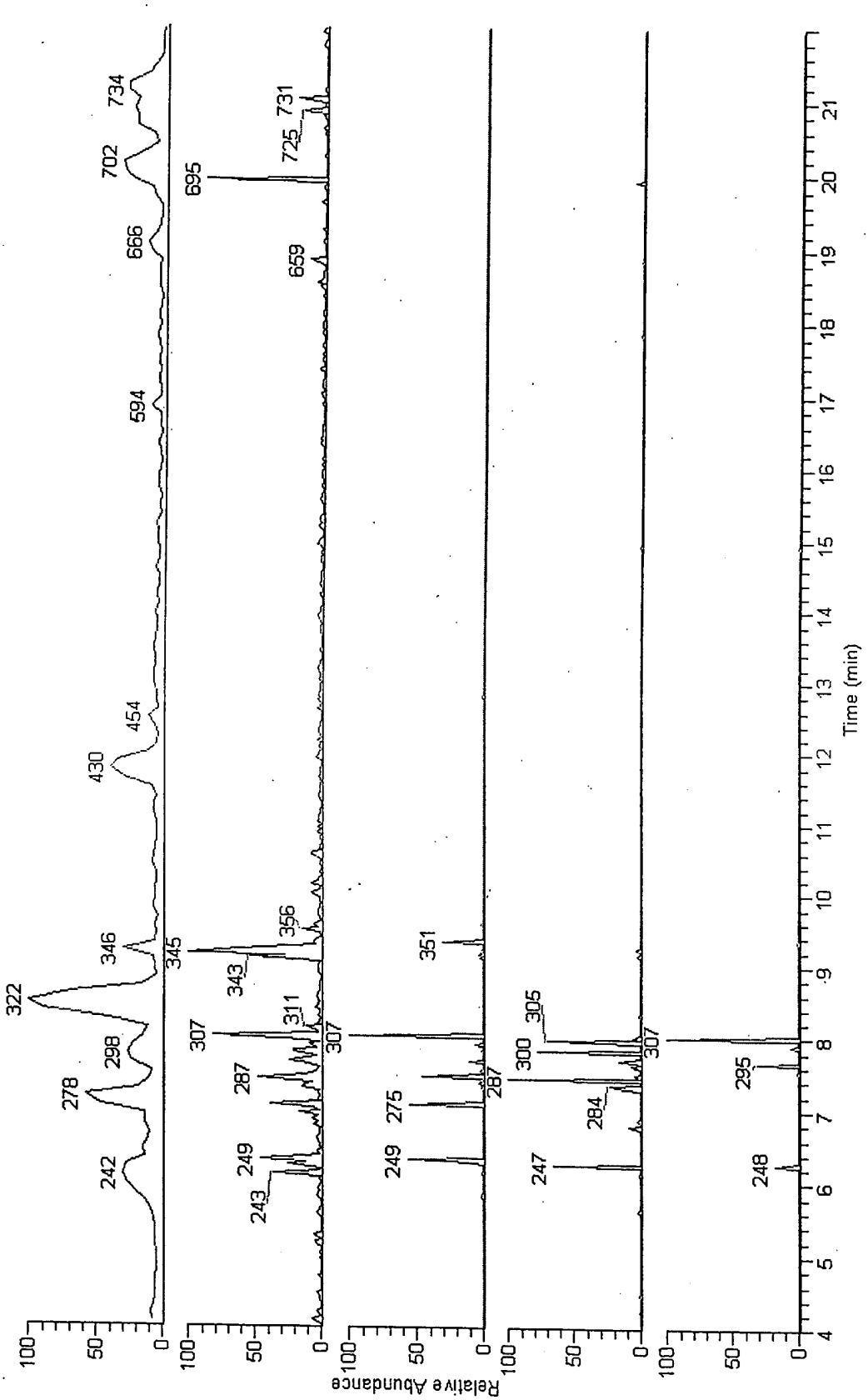

FIGURE 26: Various chromatograms obtained by LC-MS/MS analysis of the modified peptides purified from a PDGF-treated 3T3 cell extract with immobilized phospho-(Ser/Thr) Akt substrate antibody.

LC-MS/MS Spectrum No.	m/z	z	m	Number of Phosphates	Seen by MALDI	Label in MALDI	Tentative Identification
351	925.900	2	1,849.786	1	+	unlabeled	
300	752.510	3	2,254.509	1	+	2,254.460	S6 + 1 PO_4
275	1,163.430	2	2,324.846	2	+	2,324.381	peptide A
265	775.710	3	2,324.109	2	+	as above	as above
288	1,167.900	2	2,333.786	2	+	2,334.427	S6 + 2 PO_4
287	779.230	3	2,334.669	2	+	as above	as above
295	584.760	4	2,335.012	2	+	as above	as above
249	1,203.370	2	2,404.726	3	+	2,404.335	peptide A + 1 PO_4
247	802.480	3	2,404.419	3	+	as above	as above
248	602.210	4	2,404.812	2?	+	as above	as above
285	1,207.810	2	2,413.606	3	+	unlabeled	S6 + 3 PO_4
284	806.480	3	2,416.419	3	+	as above	as above
233	1,242.520	2	2,483.026	3	+	unlabeled	
227	829.130	3	2,484.369	3	+	as above	
283	949.670	3	2,845.989	3	+	unlabeled	
305	1,068.670	3	3,202.989	2			
307	802.330	4	3,205.292	2	+	unlabeled	
303	909.870	4	3,635.452	2			
296	1,239.290	3	3,714.849	3?			
293	1,265.510	3	3,793.509	4?			
289	1,292.210	3	3,873.609	3			
291	981.850	4	3,923.372	2			

For "Label in MALDI", values indicate masses as labeled in Figure 25.
Note masses above 3,600 were not measured during MALDI-TOF mass spectrometry.

FIGURE 27: Properties of the peptides that were observed to undergo neutral-loss during the LC-MS/MS analysis shown in Figure 26.

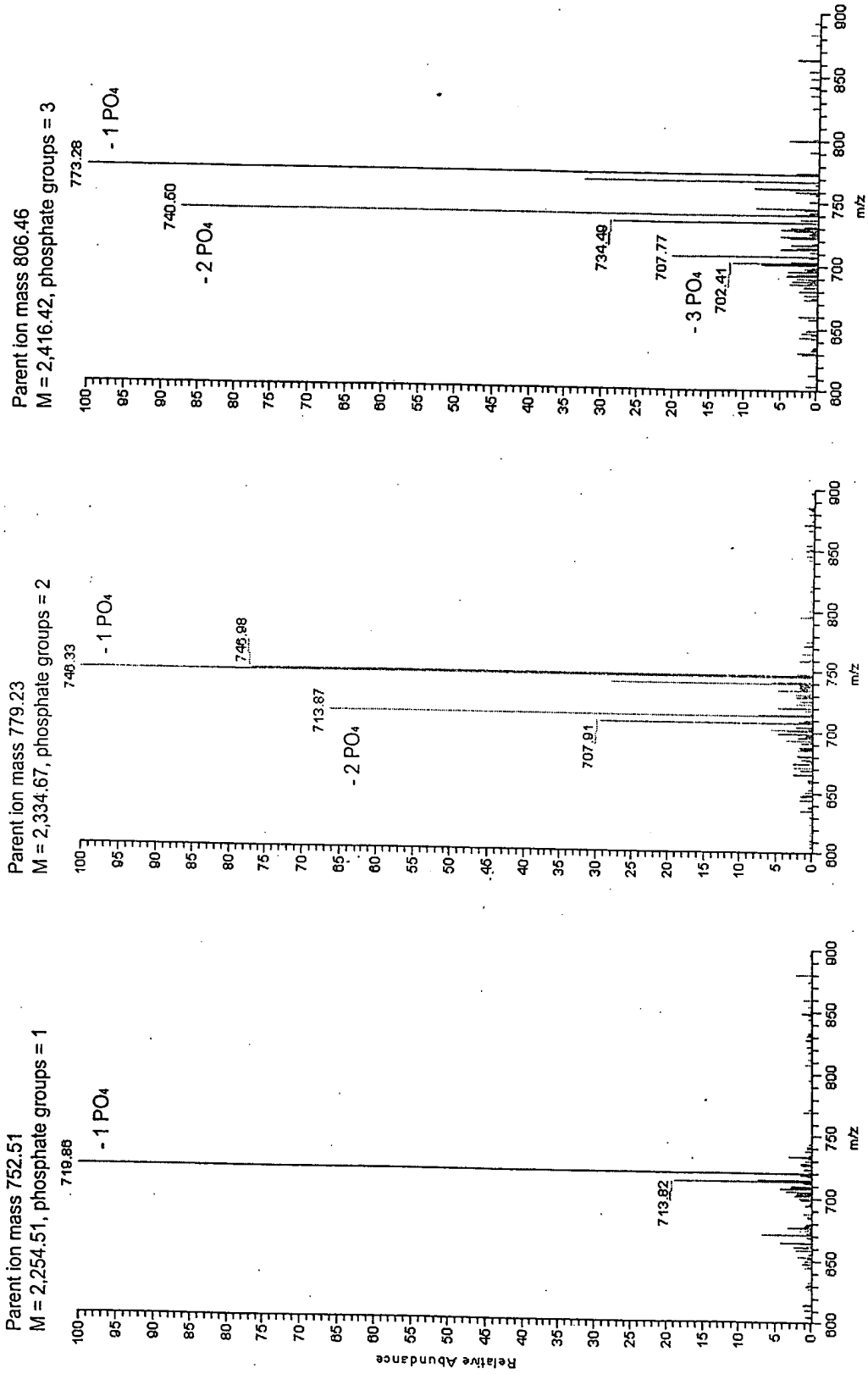

FIGURE 28: Three MS/MS spectra acquired during the LC-MS/MS analysis shown in Figure 26. These three spectra have been tentatively assigned to the multiply phosphorylated peptide from the ribosomal protein S6 with one (panel 1), two (panel 2), or three (panel 3) phosphate groups.

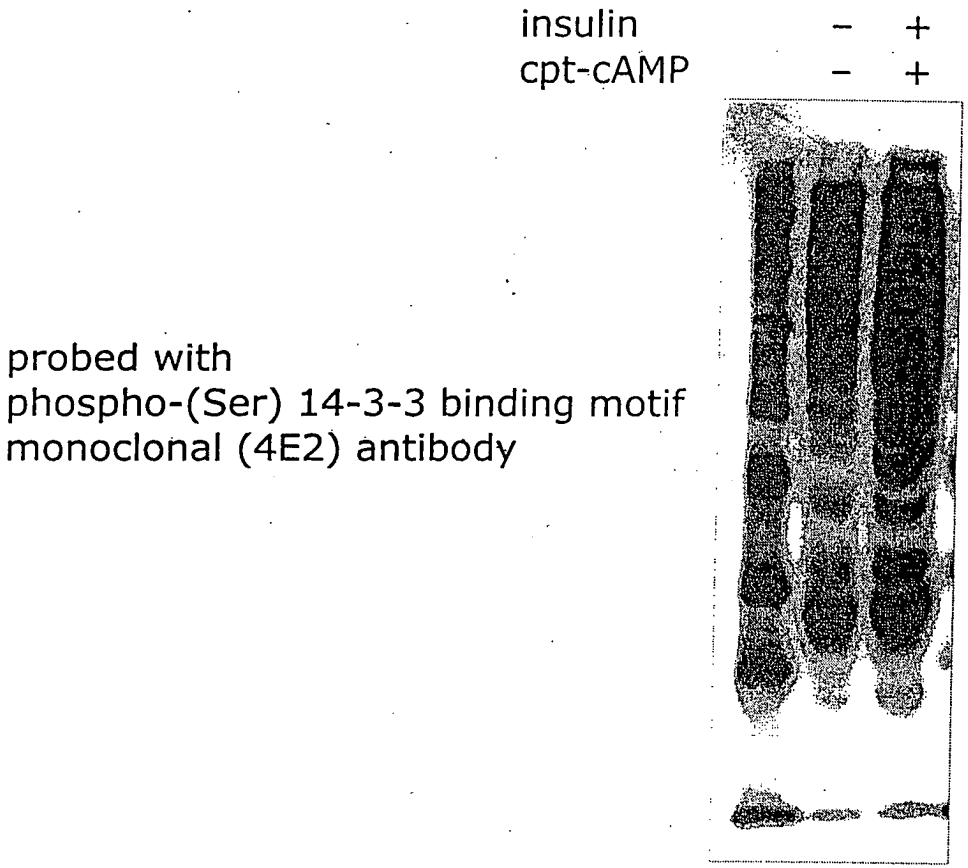

FIGURE 29: Western blot of COS-1 cells treated with insulin and an analog of cAMP and probed with phospho-(Ser) 14-3-3 binding site antibody.

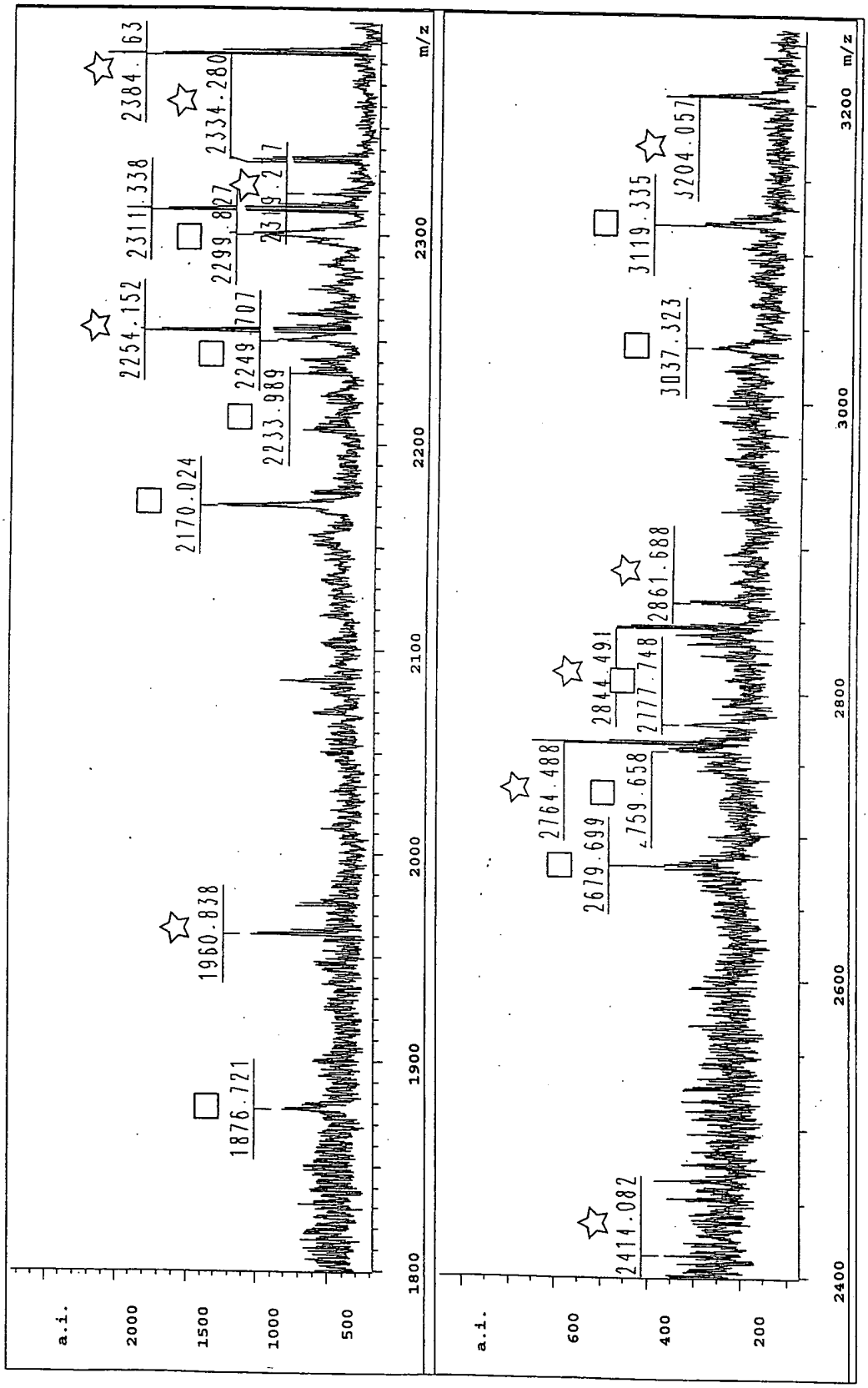

FIGURE 30: MALDI-TOF mass spectrum of modified peptides isolated from a treated COS-1 cell extract with immobilized phospho-(Ser) 14-3-3 binding site antibody.

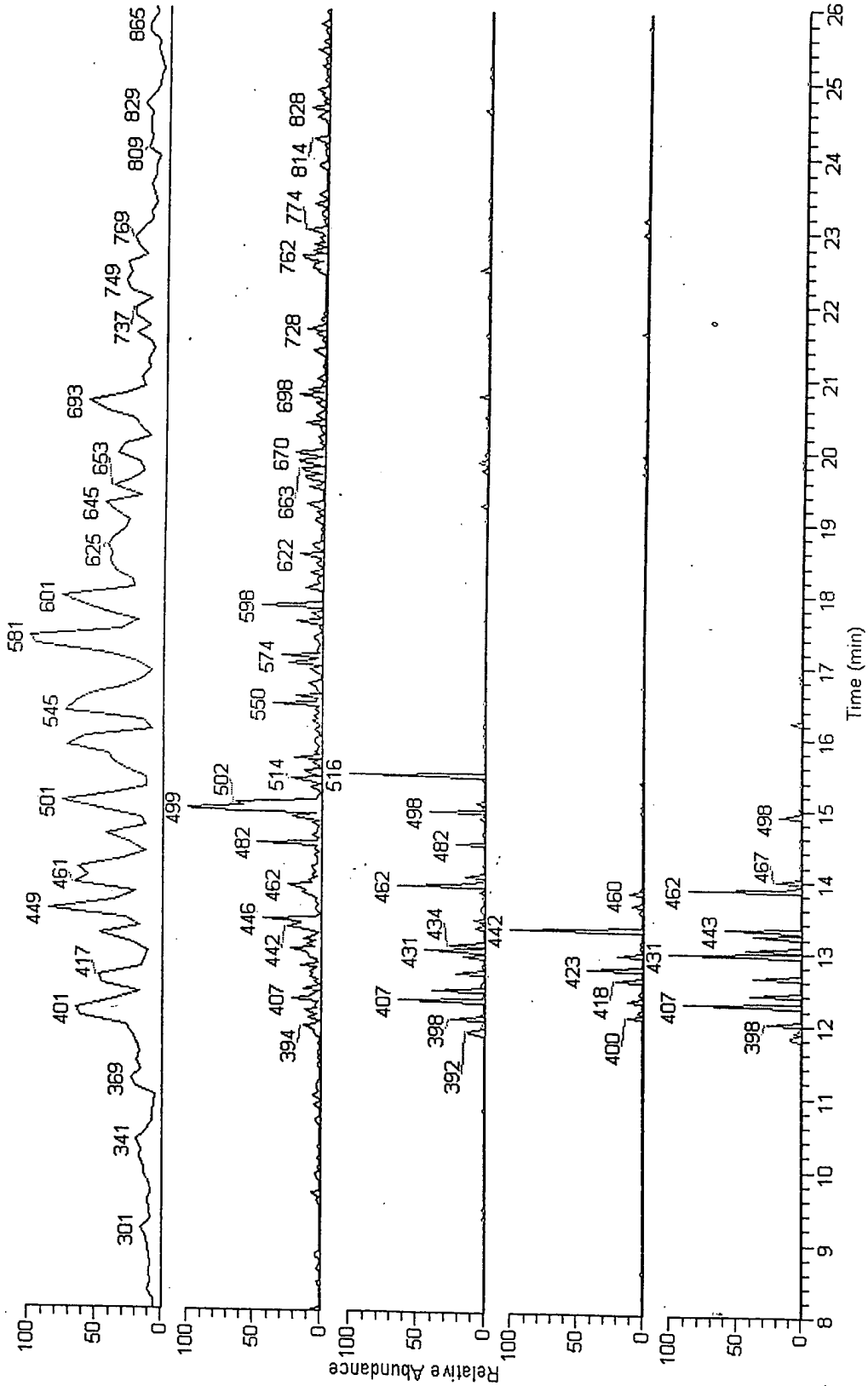

FIGURE 31: Various chromatograms obtained by LC-MS/MS spectrum of the modified peptides purified from a treated COS-1 cell extract with immobilized phospho-(Ser) 14-3-3 binding site antibody.

LC-MS/MS Spectrum No.	m/z	z	m	Number of Phosphates	Seen by MALDI	Label in MALDI	Tentative Identification	Seen by LC-MS of Akt-3T3
454	746.800	3	2,237.379	1				
442	752.540	3	2,254.599	1	+	2,254.152	S6 + 1 PO_4	+
443	564.670	4	2,254.652	1	+	as above	as above	+
436	774.990	3	2,321.949	2				
440	583.220	4	2,328.852	1				
428	1,168.360	2	2,334.706	2	+	2,334.280	S6 + 2 PO_4	+
418	779.220	3	2,334.639	2	+	as above	as above	+
420	585.030	4	2,336.092	2	+	as above	as above	+
516	1,192.610	2	2,383.206	1	+	2,384.163		
423	803.800	3	2,408.379	2				
400	805.720	3	2,414.139	3	+	2,414.082	S6 + 3 PO_4	+
398	604.780	4	2,415.092	3	+	as above	as above	+
403	830.710	3	2,489.109	3				
432	633.230	4	2,528.892	1				
430	922.510	3	2,764.509	2	+	2,764.488		
431	692.400	4	2,765.572	2				
434	710.790	4	2,839.132	2				
408	949.190	3	2,844.549	3	+	2,844.491		+
407	712.240	4	2,844.932	3	+	as above		+
412	730.590	4	2,918.332	3?				
391	732.120	4	2,924.452	3	~			
392	750.660	4	2,998.612	3?	~			
460	1,069.440	3	3,205.299	2	+	3,204.057		+
463	802.150	4	3,204.572	2	+	as above		+
462	820.490	4	3,277.932	2				
467	838.850	4	3,351.372	2				
451	1,288.970	3	3,863.889	2?				

For "Label in MALDI", values indicate masses as labeled in Figure 30.
Note masses above 3,600 were not measured during MALDI-TOF mass spectrometry.

FIGURE 32: Properties of the peptides that were observed to undergo neutral-loss during the LC-MS/MS analysis shown in Figure 31.

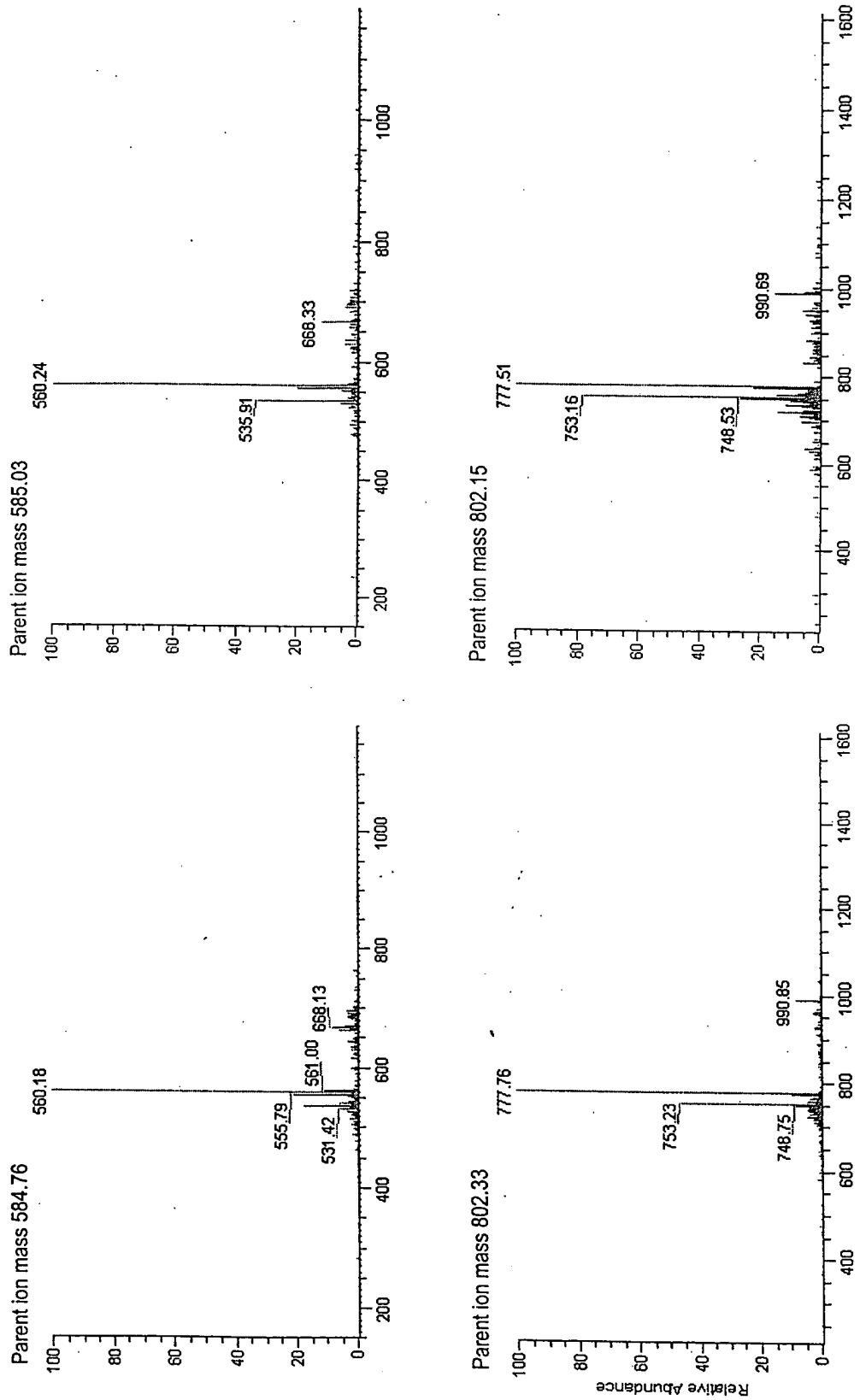

FIGURE 33: Two MS/MS spectra acquired during the LC-MS/MS analysis of two different samples, one prepared with phospho-(Ser/Thr) Akt substrate antibody (Figure 26) (left panels of this figure), the other prepared with phospho-(Ser) 14-3-3 binding site antibody (Figure 31) (right panels of this figure).

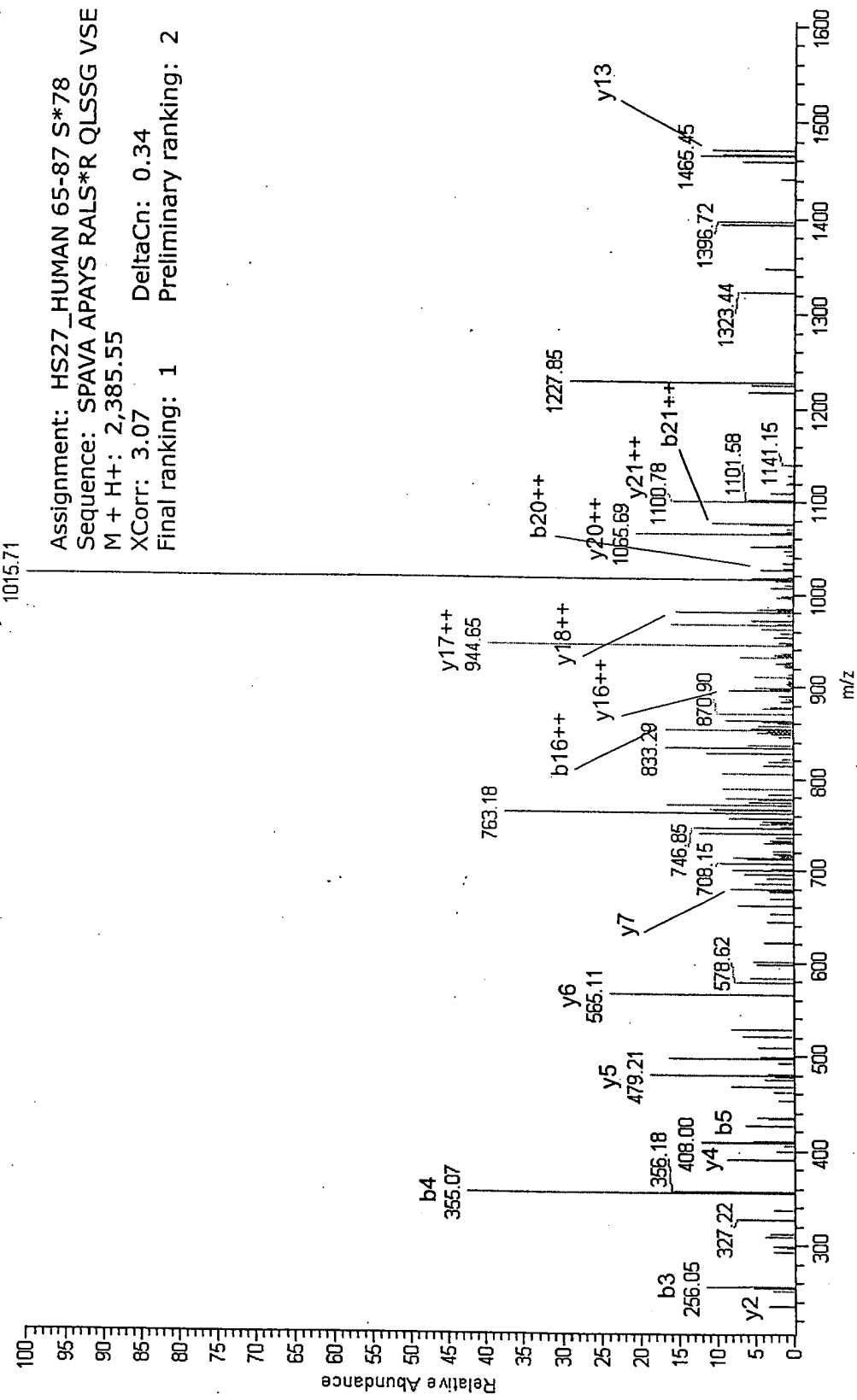

FIGURE 34: An LC-MS/MS spectrum of one of the modified peptides purified from a treated COS-1 cell extract with immobilized phospho-(Ser) 14-3-3 binding site antibody.